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Lignin/Hydroxycinnamic Acid/Polysaccharide Complexes: Synthetic Models for Regiochemical Characterization

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The effective separation or utilization of plant cell wall carbohydrates is often thwarted by its intimate association with the phenolic polymer, lignin. Considerable effort has been expended to identify the relatively few covalent bonds that exist between carbohydrates and lignins in woody plants (Reviewed in: Jeffries, 1990). In forage legumes and grasses, this lignin component is also covalently linked to phenolic acids, notably the 4-hydroxycinnamic acids *p*-coumaric acid and ferulic acid (Fig. 9–1) (Reviewed in: Monties, 1991; Yamamoto et al., 1989; Fry & Miller, 1989). Indigestible itself, this lignin-phenolic acid complex further inhibits the digestion, by ruminants, of potentially digestible carbohydrates (Reviewed in: Jung & Ralph, 1990; Hartley & Ford, 1989; Buxton & Russell, 1988; Jung & Fahey, 1983). For example, long-term in vitro digestion of forage materials results in indigestible residues which typically contain 60 to 70% carbohydrates (D.R. Mertens and R.D. Hatfield, 1992, unpublished data). Analysis shows that the component carbohydrate monomer composition is surprisingly similar to that of the original feed. It is to obtain an understanding of the structural factors that limit digestibility that many researchers are now pursuing studies involving the cross-linking of carbohydrates and lignin polymers by phenolic acids.

Cross-linking can have a dramatic effect on the properties of a polymer. A familiar example is the disulfide bridge cross-linking of rubber by vulcanizing that turns natural rubber from a soft, highly solvent-swellable polyisoprene polymer into a durable material that survives as automotive tires. Researchers have postulated the effects of cross-linking on plant cell wall properties such as accessibility, extensibility, plasticity, and digestibility (Taiz, 1984; Fry, 1984; Reviewed in: Jung & Ralph, 1990; Fry & Miller, 1989; Yamamoto et al., 1989; Hartley & Ford, 1989).

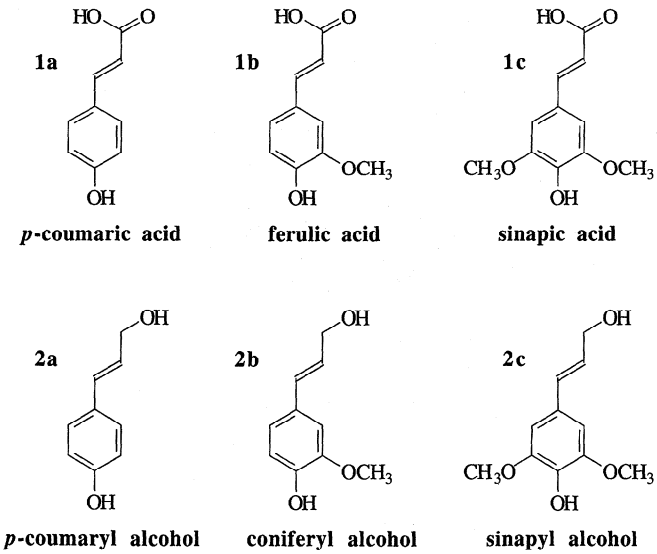


Fig. 9-1. Hydroxycinnamic acids *p*-coumaric acid **1a**, ferulic acid **1b**, and sinapic acid **1c**, and their derived hydroxycinnamyl alcohol lignin precursors; *p*-coumaryl alcohol **2a**, coniferyl alcohol **2b**, and sinapyl alcohol **2c**.

Due to the complexity of the cell wall system, the large variety of possible component interactions, and the present inability to obtain sufficiently detailed structural information on intact systems, heavy use is made of model systems and cell wall isolates. The use of model systems is extremely valuable in this area: high-resolution spectroscopic data can be obtained from structures of interest, ^{13}C NMR chemical shifts in appropriate low molecular weight compounds are identical to those in the polymer (Ralph, 1982a,b), reactivity data that would be difficult to extract from reactions on the polymer system are readily available from models, and synthetic strategies for manipulation of cell wall components can be worked out and optimized. Additionally, available pathways for cell wall intermediates can be elucidated. An example is in determining the pathways available for ferulic acid during the free-radical catalyzed lignification (to be discussed below). Finally, since specific labeling of model compounds is possible and components may be used at higher loadings, enhanced mechanistic detail may be revealed. With the judicious use of model compounds, it is hoped to extend the concept of the plant cell wall far beyond the broad compositional picture to a more detailed structural and molecular description. Armed with this knowledge it becomes possible to target plants' genetic traits that are desirable/undesirable for selection processes or genetic manipulation, and will allow full use to be made of results of the proposed plant genome projects. Other aspects of improved digestibility are discussed in other chapters of this book.

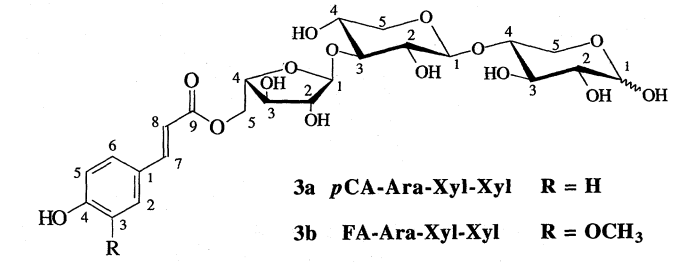
Excellent reviews are available that describe lignin and lignification in legumes and grasses (Yamamoto et al., 1989; Monties, 1991; Delmer & Stone, 1988; Bacic et al., 1988; Hartley & Ford, 1989; Lapierre, see chapter 6 in

this book) freeing this section from an extensive review of cell wall components. Hence, the goal of this chapter is to introduce current work using model compounds aimed at obtaining detailed structural information that will subsequently help to identify promising ways of extending the molecular description of the plant cell wall in forage plants.

I. LINKAGES BETWEEN POLYSACCHARIDES AND HYDROXYCINNAMIC ACIDS

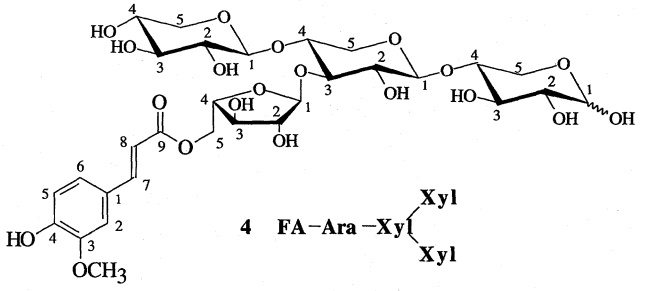
The association of ferulic acid and *p*-coumaric acid with polysaccharides has been extensively investigated (Reviewed in: Jung & Ralph, 1990). Partial enzymatic hydrolyses of various grass cell walls and subsequent chromatographic isolation have afforded hydroxycinnamic acids attached, as esters, to diagnostic carbohydrate fragments. These low molecular weight compounds (Fig. 9–2) have been amenable to detailed structural characterization by NMR. Compounds FA-Ara-Xyl, *p*CA-Ara-$(Xyl)_2$ **3a**, FA-Ara-$(Xyl)_2$ **3b**, FA-Ara-$(Xyl)_3$ **4** (Fig. 9–2), have all been isolated and characterized from enzymatic hydrolysis products of bamboo shoots (Ishii & Hiroi, 1990a,b; Ishii et al., 1990), coastal bermudagrass (Hartley et al., 1990b), bagasse (Kato et al., 1983, 1987), barley straw (Mueller-Harvey et al., 1986), corn (Kato & Nevins, 1985), or wheat bran (Smith & Hartley, 1983). In each case, the hydroxycinnamic acid is esterified to the primary hydroxyl of the arabinose moiety, which itself is linked to the 3-position of the xylose (the initially reported 2-attachment in FA-Ara-Xyl [Smith & Harley, 1983] was in fact 3, and is consistent with the xylose-homologues **3b** and **4**). An acetyl derivative, with a 2-acetoxy group on the arabinosyl moiety of FA-Ara-Xyl-Xyl **3b**, has also been recently isolated (Ishii, 1991). Mueller-Harvey et al. (1986) estimated that for barley one in every 31 arabinose residues was esterified with *p*-coumaric acid and one in every 15 with ferulic acid. Ishii et al. (1990) have also isolated FA-Xyl-Glc **5** indicating that attachment of ferulic acid also occurs to secondary hydroxyls of xylose in xyloglucans. Secondary esters have also been reported onto galactose, in 4-*O*-(6-*O*-feruloyl-β-D-galactopyranosyl)-D-galactose and arabinopyranose, in 3-*O*-(3-*O*-feruloyl-α-L-arabinopyranosyl)-L-arabinose), in spinach pectins (Fry, 1982). These structural assignments were from electrophoretic mobilities and other reaction data; unambiguous NMR data are not available.

These isolated compounds have established the nature of the bonding and individual carbohydrate monomers (and consequently the polymers) involved. However, for various spectroscopic, enzymatic, structural and reactivity studies, it has been necessary to obtain well-characterized compounds of this type in larger quantities than are readily available from isolation methods. To synthesize compounds containing hydroxycinnamic acids and representative carbohydrate oligomers such as arabinoxylans, two major synthetic methodologies had to be developed. Firstly, the synthesis of pentose sugar oligomers possessing the desired regiochemistry has been troublesome or unknown. As described below, new general regiospecific protection strate-

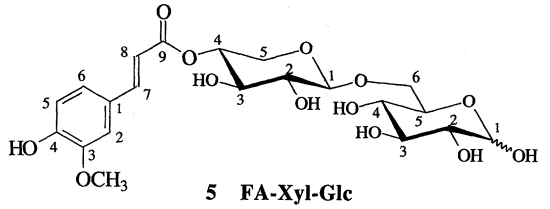


3a *p*CA-Ara-Xyl-Xyl R = H

3b FA-Ara-Xyl-Xyl R = OCH_3



4 FA–Ara–Xyl(Xyl)(Xyl)



5 FA-Xyl-Glc

Fig. 9-2. Hydroxycinnamic acid/carbohydrate oligomers isolated from grass cell wall enzyme hydrolysates. Primary esters *O*-[5-*O*-(*trans*-*p*-coumaroyl)-α-L-arabinofuranosyl]-(1→3)-*O*-β-D-xylopyranosyl-(1→4)-D-xylopyranose (*p*CA-Ara-Xyl_2) **3a**, *O*-[5-*O*-(*trans*-feruloyl)-α-L-arabinofuranosyl]-(1→3)-*O*-β-D-xylopyranosyl-(1→4)-D-xylopyranose (FA-Ara-Xyl_2) **3b**, *O*-β-D-xylopyranosyl-(1→4)-*O*-[5-*O*-*trans*-feruloyl-α-L-arabinofuranosyl-(1→3)]-*O*-β-D-xylopyranosyl-(1→4)-D-xylopyranose (FA-Ara-Xyl_3) **4**. Secondary ester *O*-[4-*O*-*trans*-feruloyl-β-D-xylopyranosyl]-(1→6)-D-glucopyranose (FA-Xyl-Glc) **5**

gies for xylopyranosides allows any of the desired isomers to be obtained. Secondly, regiospecific esterification of the carbohydrate by the hydroxycinnamic acid was also required, demanding new regioselective protection/deprotection strategies.

A. Regioselective Protection Strategies for Xylopyranosides

Development of a regioselective protection strategy for D-xylopyranosides (Helm et al., 1991) has significantly decreased the number of steps required to provide suitable precursors for oligosaccharide production. In some cases, the number of steps can be reduced from six to one with a concomitant major improvement in yield.

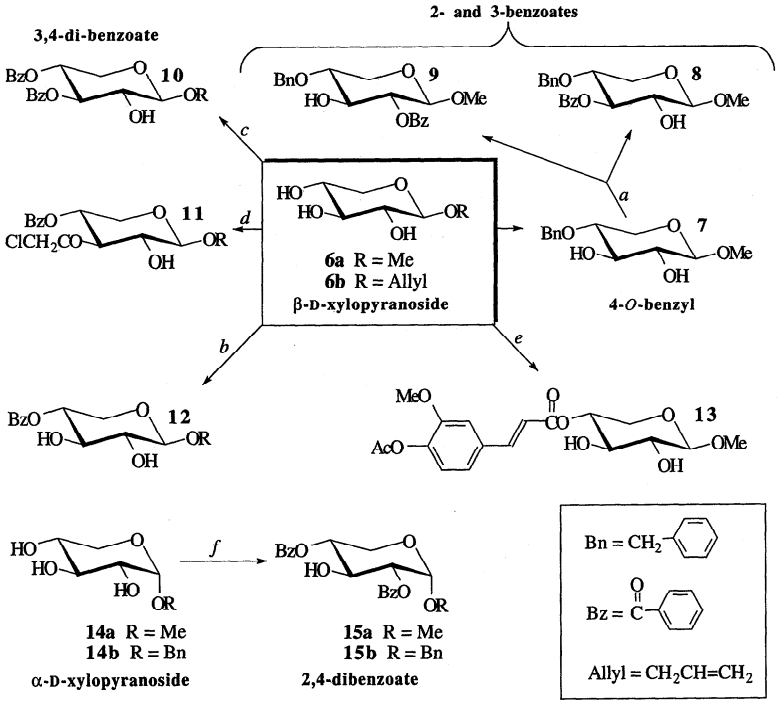


Fig. 9-3. The use of dibutyltin oxide for the protection of D-xylopyranosides. All reactions involve initial formation of the stannylene acetal using 1 eq. of dibutyltin oxide and then: (a) 1 eq. BzCl, yield of **8** + **9** 85%; (b) 1 eq. BzCl, yield 80%; (c) 2 eq. BzCl, yield 90%; (d) 1 eq. BzCl followed by 1 eq. chloroacetyl chloride, yield 53%; (e) 1 eq. feruloyl chloride, yield 59%; (f) 2 eq. BzCl, yield 82+%. All yields are of purified isolated products that are generally crystalline (Helm et al., 1991).

Methyl β-D-xylopyranoside **6a** (Fig. 9-3) can be converted to the 4-*O*-benzyl derivative **7** in high yield. This compound, when treated with dibutyltin oxide, formed a mixture of stannylene acetals. Stannylene acetals "activate" one of the ring oxygens by forming a dimer, such as the one represented in Fig. 9-4, the type being determined by relative stabilities. Predicting the activated site is generally not possible and must be determined experimentally. When activated, the oxygen of the stannylene acetal will react with acyl donor compounds, such as benzoyl chloride to form a benzoate. Stannylation of the 4-*O*-benzyl compound **7** (Fig. 9-3) followed by addition of one equiva-

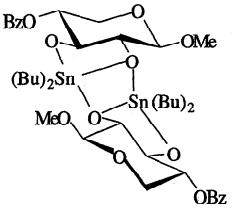


Fig. 9-4. A stannylene acetal dimer where the 3-hydroxyl is activated.

lent of benzoyl chloride gave about equal amounts of the easily separable 3- and 2-benzoates **8** and **9** in a combined yield of 85%. These two compounds have only one hydroxyl remaining and can be used in preparing 2- or 3-linked disaccharides.

The utility of dibutyltin oxide-mediated reactions for generating protected D-xylopyranosides was further demonstrated during an investigation of the activation of alkyl α- and β-D-xylopyranosides (Fig. 9–3). Both methyl **6a** and allyl **6b** β-D-xylopyranosides provided mono- or dibenzoates depending on the amount of benzoyl chloride added. The addition of one equivalent of benzoyl chloride gave the 4-benzoate **12** (80% yield). A reaction with two equivalents of benzoyl chloride furnished the 3,4-di-benzoate **10** in 90% yield. The clean conversion to mono- or di-esters, depending on the amount of acyl chloride added, allowed differentiation between the 3- and 4-positions and thus synthesis of a 4-*O*-feruloyl derivative **13** (which depicts the ester linkage recently identified by Ishii et al. [1990]), as well as a 3-*O*-chloroacetyl-4-*O*-benzoyl derivative **11**. The chloroacetyl group can be removed without removing the benzoate, and therefore the reaction has not only protected (in a regioselective fashion) the 3- and 4-positions but has also differentiated between these two hydroxyls. The attractiveness of this scheme is that any of these selectively derivatized compounds can be obtained in a single step. Both methyl **14a** and benzyl **14b** α-D-xylopyranosides afforded the 2,4-di-*O*-benzoates, **15a** and **15b**, with the addition of two equivalents of benzoyl chloride. There was no selectivity obtained when only one equivalent was added. However, the acylation of the 2- and 4-positions complements the results obtained with dibutyltin oxide-mediated benzoylation of the β-D-xylopyranosides, and a combination of the two reactions allows substitution at any desired site.

B. Synthesis of Arabinoxylan Oligosaccharides

A key compound necessary for arabinoxylan synthesis is an L-arabinofuranosyl halide such as **17** (Fig. 9–5). This material can be condensed with a D-xylopyranosidic hydroxyl affording a glycosidic bond (a glycosylation reaction). The standard technique provides an unstable material in only 36% yield (Fletcher, 1963). An improved method (Helm et al., 1991) converts methyl α-L-arabinofuranoside tribenzoate **16** (Fig. 9–5) to the corresponding chloride **17** by the action of dichloromethyl methyl ether and tin tetrachloride. The reaction affords the crystalline α-L chloride in 85% yield. The material is stable and is thus an excellent alternative to the earlier technique.

For the preparation of protected β-D-xylopyranosides, the use of dibutyltin oxide proved indispensable. The formation of 3- and 2-benzoates **8** and **9** with a benzyl group in the 4-position is shown in the upper scheme of Fig. 9–3 (Helm et al., 1991). The use of standard coupling techniques (silver triflate and collidine, Fig. 9–5) afforded the α-L-linked disaccharides in high yields (>90%). The scheme for the 3-benzoate **8** to give the 2-linked disaccharide **18** is the top scheme in Fig. 9–5. There are two possible products from a glycosylation reaction—the desired α-L-linked dimer and the β-L-linked

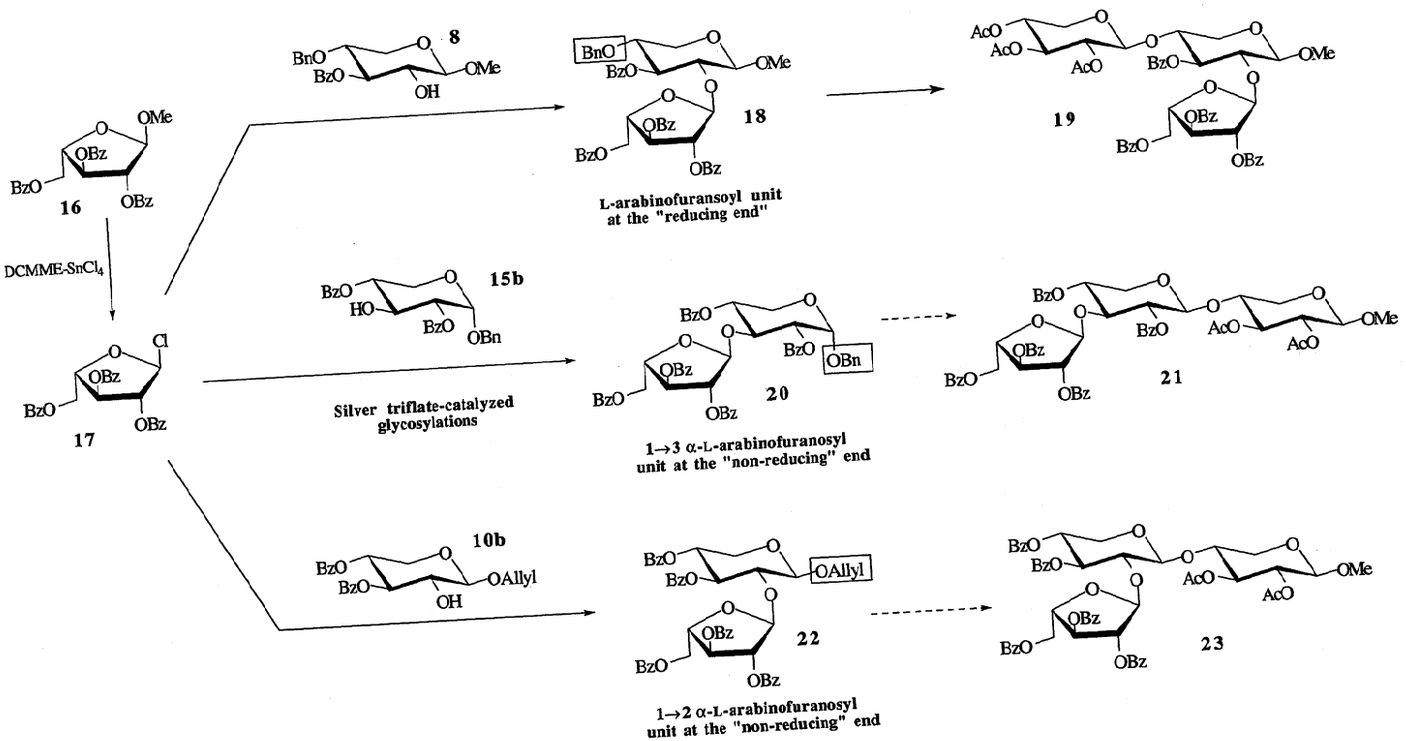


Fig. 9-5. Regioselective approaches to arabinoxylan oligosaccharides.

dimer. Conditions were developed to minimize formation of the β-L-linkage. The placement of the benzyl group at the four-position was important because it could be removed selectively under mild conditions. The debenzylation reaction occurred in high yield (90%) and exposed the 4-hydroxyl for eventual glycosylation with a xylopyranosyl halide. In this way, the two arabinoxylan trisaccharides with the arabinofuranosyl moiety at the reducing end can be prepared; for example, **19** from **18** in the top scheme in Fig. 9–5.

Preparation of the arabinoxylan disaccharides with the α-L-arabinofuranosyl moiety 1→3 linked at the nonreducing end (Fig. 9–5, middle scheme) was accomplished by preparation of the 2,4-di-*O*-benzoate **15b** (Fig. 9–3) from benzyl α-D-xylopyranoside and dibutyltin oxide-mediated benzoylation. Standard glycosylation of **17** with **15b** afforded the appropriate disaccharide **20** in 89% yield. Removal of the benzyl group, conversion to the corresponding halide, and subsequent condensation would afford the trisaccharide **21**. The remaining required linkage, present in compound **22** (Fig. 9–5, bottom scheme) can be accomplished by condensation of the arabinofuranosyl chloride **17** with allyl 3,4-di-*O*-benzoyl-β-D-xylopyranoside **10b** (available from dibutyltin oxide activation in one step, Fig. 9–3). Removal of the allyl group from the disaccharide **22** and subsequent coupling would provide the desired trisaccharide **23** (R.F. Helm and J. Ralph, 1991, unpublished data).

C. Preparation of Hydroxycinnamic Acid—Carbohydrate Esters

Since the typical location of feruloyl and *p*-coumaroyl substituents on polysaccharides is the primary position of α-L-arabinofuranosyl residues, we sought to prepare a series of hydroxycinnamoyl-carbohydrate esters with substitution of the primary hydroxyl. The compounds could then be used to assess esterase activity in enzyme preparations, plant tissues, or rumen fluid (Hatfield et al., 1991, 1993).

The initial preparations used the higher kinetic selectivity of the esterification reaction for the primary over the secondary alcohols (Hatfield et al., 1991a). However, the low yields of desired products and contamination with secondary acylation products deemed it unsuitable for use in the synthesis of models that contained ^{13}C-labeled ferulic acid.

Improved procedures (Helm et al., 1992) involve selective protection strategies (Fig. 9–6). The primary positions of methyl β-D-glucopyranoside **24** and methyl β-D-galactopyranoside **27** were readily available for condensation by complete trimethylsilylation followed by selective de-*O*-silylation of the primary position with methanolic potassium carbonate (top scheme of Fig. 9–6). Subsequent coupling with 4-acetoxy feruloyl and *p*-coumaroyl chlorides gave the desired protected esters in good yields (80% overall for the D-galactopyranoside esters **29**). Traditional de-protection gave the desired hydroxycinnamoyl pyranoside esters **26** and **29**. This procedure was not successful for methyl α-L-arabinofuranoside **30**, and a different reaction scheme was developed. A one-pot *t*-butyldimethylsilylation/acetylation reaction

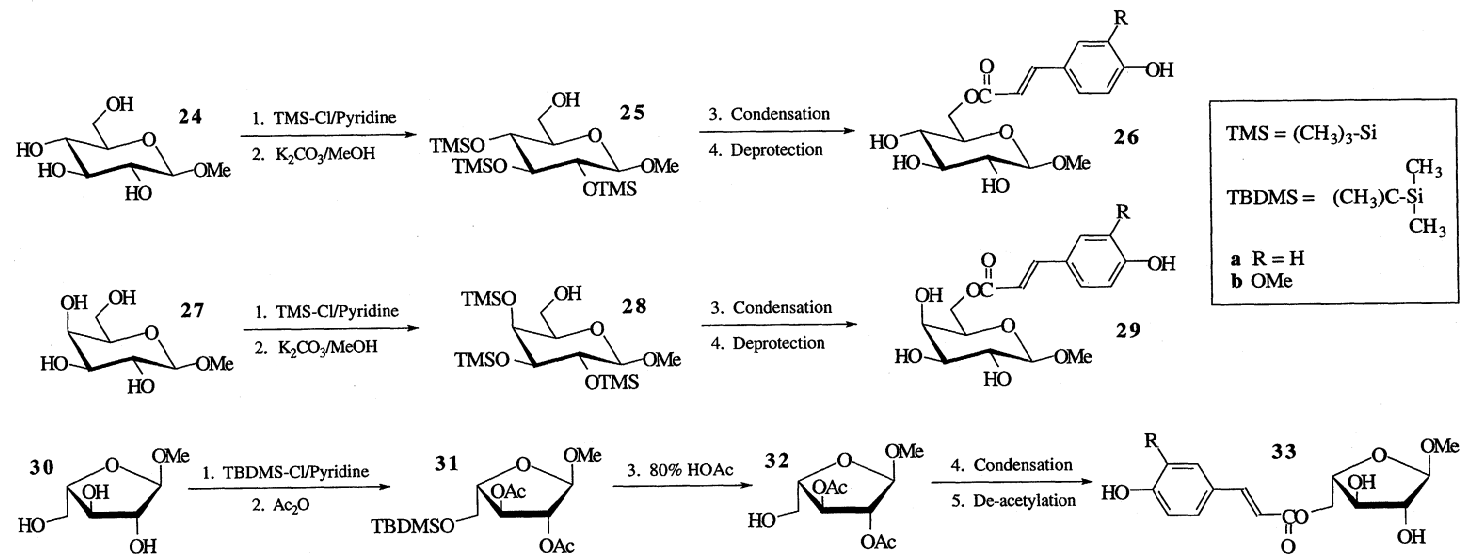


Fig. 9-6. Synthetic scheme for *p*-coumaroyl and feruloyl esters of furanosides and pyranosides.

provided selective protection of the primary hydroxyl to give **31** (lower scheme, Fig. 9–6). Cleavage of the *t*-butyldimethylsilyl (TBDMS) group with 80% aqueous acetic acid gave **32** with the 5-hydroxyl exposed for condensation. Condensation with the hydroxycinnamoyl chlorides and subsequent de-acetylation with pyrrolidine in 95% ethanol gave the hydroxycinnamoyl esters **33** in high yields.

Figure 9–7 shows the long-range ^{13}C-^{1}H correlation spectrum of methyl 5-*O*-feruloyl-α-L-arabinofuranoside **33b**. This experiment, which maximizes the interaction between carbons and protons that are two to three bonds apart, is useful in structural elucidation. Of particular note is the correlation between C-9 and one of the H-5′ protons confirming esterification at the primary site. The carbons without attached protons were assigned from the correlations of the 3-methoxyl protons to C-3, the 4-OH to C-5 and C-3, and the H-8 and H-5 protons to C-1.

Since FA-Ara (methyl 5-*O*-feruloyl-α-L-arabinofuranoside, **33b**) is such a valuable substrate for a variety of studies (incorporation into DHPs; assessment of esterase activities, incorporation into growing plants), an improved synthesis was developed (Ralph et al., 1992). This synthetic scheme minimized reaction intermediate isolation and provided FA-Ara **33b** in 60% overall yield based on ^{13}C-labeled ferulic acid.

Table 9–1 (Helm et al., 1992) lists ^{13}C NMR data for the feruloylated and *p*-coumaroylated methyl glycosides of β-D-gluco- and β-D-galactopyranose, and α-L-arabinofuranose. Unambiguous assignments were based on short- and long-range heteronuclear ^{13}C-^{1}H shift-correlated experiments. It is apparent that the feruloyl sidechain carbons (C-7, C-8, and C-9) are relatively insensitive to the carbohydrate and aryl moieties and also, from other data (Helm et al., 1992) to acetylation. The C-7 carbon resonances range from 144.8 to 146.1 for samples in acetone-d_6. The C-8 carbon resonances behave in a similar fashion although there is a significant difference in the frequencies of the acetylated vs. free compounds (118.9–118.2 vs. 115.5–115.2, respectively). Feruloylated glycosides **26b**, **29b**, and **33b** exhibited C-8 resonances up-field of C-5 (115.5 vs. 116.0 in acetone-d_6; 114.9 vs. 115.9 in acetone-d_6:D_2O, 9:1) which confirms, as recognized by Ishii and Hiroi (1990a), that all previous assignments for these two carbons were incorrect and should be interchanged.

II. STRUCTURE OF FORAGE LIGNIN/HYDROXYCINNAMIC ACID COMPLEXES

Lignins in herbaceous plants are syringyl-guaiacyl (derived from both coniferyl **2b** and sinapyl alcohol **2c** monomers, Fig. 9–1) lignins, more closely analogous to the lignins in angiosperm woods than in gymnosperms (Sarkanen & Ludwig, 1971; Fengel & Wegener, 1989). One feature that distinguishes lignins in forage plants is the presence, intimately associated with cell wall components, of the hydroxycinnamic acids *p*-coumaric acid **1a**, ferulic acid **1b**, and sinapic acid **1c**, Fig. 9–1. These three acids are precur-

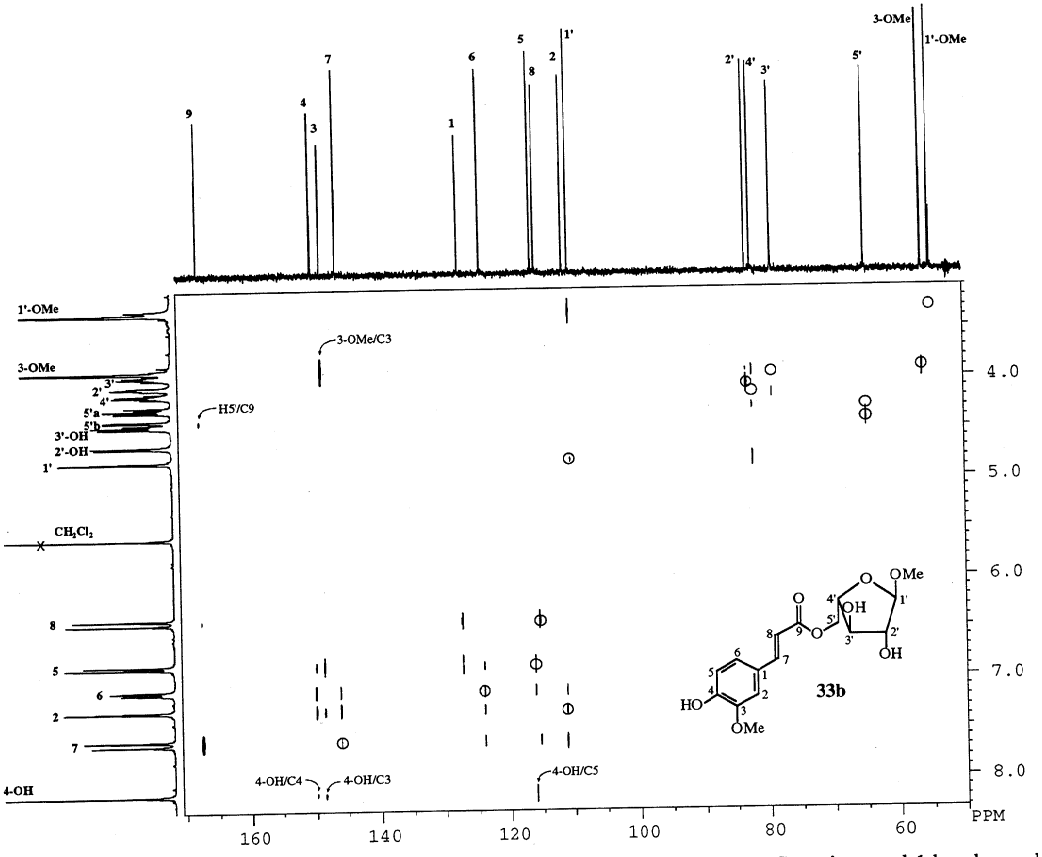


Fig. 9-7. Long-range ^{13}C-^{1}H correlation spectrum of methyl 5-*O*-feruloyl-α-L-arabinofuranoside **33b**. Superimposed 1-bond correlation data is schematically indicated by open circles. Taken from Hatfield et al., 1991a.

Table 9-1. ^{13}C NMR chemical shifts (ppm) for selected compounds.†

	D-Glucopyranosyl		D-Galactopyranosyl		L-Arabinofuranosyl	
Carbon	**26a**	**26b**	**29a**	**29b**	**33a**	**33b**
1'-OMe	56.7	56.7	56.6	56.6	55.0	56.3
3-OMe	--	56.3	--	56.3	--	55.0
1'	104.8	104.8	105.2	105.3	110.2	110.3
2'	74.5	74.6	72.0	72.0	83.1	83.2
3'	77.6	77.7	74.4	74.4	79.2	79.3
4'	71.2	71.2	69.7	69.6	82.3	82.4
5'	74.7	74.8	73.3	73.3	64.7	64.8
6'	64.2	64.2	64.0	64.0	--	--
1	126.7	127.3	126.6	127.3	126.8	127.4
2	130.9	111.2	130.9	111.3	130.9	111.2
3	116.6	148.7	116.7	148.7	116.6	148.7
4	160.6	150.0	160.5	150.1	160.5	150.1
5	116.6	116.0	116.7	116.0	116.6	116.0
6	130.9	124.0	130.9	124.0	130.9	124.1
7	145.6	145.9	145.6	146.0	145.7	146.1
8	115.1	115.4	115.2	115.5	115.2	115.5
9	167.6	167.6	167.4	167.4	167.4	167.4

† In acetone-d_6.

sors to *p*-coumaryl alcohol **2a**, coniferyl alcohol **2b** and sinapyl alcohol **2c** that are dehydrogenatively polymerized to form lignins (Adler, 1977; Harkin, 1967, 1973). That these acid monomers are covalently linked to cell wall polymers is indicative of the different biochemical processes occurring in herbaceous plants versus woody plants.

In herbaceous plants, lignin cannot be considered in isolation from the phenolic acid components. The working definitions of "core" and "noncore" lignins (see chapter 13 by Jung and Deetz in this book) have been used to describe the material left after hydrolysis and the hydroxycinnamic acid components released during hydrolysis, respectively. Although these definitions are convenient for investigations into the nutritional aspects of forage, as well as for comparing plant compositions, the terminology is quite unsound with respect to discussion of the molecular aspects of lignin structure. As will be seen shortly, only some of the hydroxycinnamic acid components that are covalently bound to cell wall polymers are released during hydrolysis, and it is this fraction that is termed "noncore" lignin. Consequently, hydroxycinnamic acids that have been incorporated into lignin with hydrolytically resistant linkages would then, by definition, be termed "core-lignin." As a further complication, there is currently no distinction between hydroxycinnamic acids attached to lignin or carbohydrates (Gordon, 1975). The use of the core/noncore terminology is misleading and inappropriate and will therefore not be used further in this chapter, which seeks to address the regiochemical aspects of hydroxycinnamic acid attachment. Instead, the complex consisting of lignin and covalently bound hydroxycinnamic acids will be termed the lignin/hydroxycinnamic acid complex and, later on, the term lignin/hydroxycinnamic acid/carbohydrate complex will be used to describe

the corresponding complex that additionally incorporates covalently bound carbohydrates.

A. Hydroxycinnamic Acids Associated with Lignins

Smith (1955) first identified ester-linked *p*-coumaric acid in a lignin preparation from wheat straw. Higuchi's group reported the presence of *p*-coumaric acid in grasses and carried out a detailed study on *p*-coumaric acid in bamboo lignins (Higuchi et al., 1967; Shimada et al., 1971; Nakamura & Higuchi, 1976, 1978a,b). The resistance of *p*-coumarate esters in bamboo to methanolysis led them to suggest that *p*-coumaric acid was linked to the γ-position of the lignin sidechain rather than the α-position.

Himmelsbach and Barton (1980) identified phenolic esters in both warm- and cool-season grasses by ^{13}C NMR. Nimz et al. (1981) examined milled lignins from bamboo (*Bambusa* spp.) and wheat straw (*Triticum aestivum* L.) by ^{13}C NMR, and found evidence for both esterified and etherified *p*-coumaric acid, supporting earlier work (Erickson et al., 1973) suggesting the presence of etherified structures. Monties' group (Scalbert et al., 1985, 1986; Sharma et al., 1986) later established the presence of ether linkages between phenolic acids and lignin fractions from wheat straw. After complete hydrolysis of esters with NaOH, a further release of phenolic acids was quantified by acidolysis, a technique traditionally used to cleave ether bonds in lignin itself. ^{13}C NMR spectra indicated the presence of both etherified and esterified phenolic acids, although the former only appeared in fractions treated with alkali. A study of phenolic acids in normal rice and a mutant brittle stem rice cultivar showed higher levels of esterified *p*-coumaric acid and markedly lower etherified ferulic acid in the mutant. Model studies confirmed that hydroxycinnamic acids could add to the α-position of quinone methides (intermediates formed during lignification) but no evidence for the regiochemistry of phenolic acids on lignin was presented.

Stone's group has improved methods for distinguishing etherified from esterified structures (Iiyama et al., 1990; Lam et al., 1990), and has recently developed novel methodology to quantitatively distinguish hydroxycinnamic acids that are ester linked, ether linked and, most importantly, both ester and ether linked (Lam et al., 1991, 1992). This work provides conclusive evidence for the surmised cross-linking function of hydroxycinnamic acids and provides some insight into the biochemical roles. Their method (Lam et al., 1991, 1992) is shown in Fig. 9–8 (an extension of their Fig. 1). Ester linkages (in **34** and **41**) can involve either lignin or polysaccharides, while ether linkages (in **38** and **41**) involve only lignin. (This assumes that phenolic glycosides of hydroxycinnamic acids are not prevalent in these cell wall fractions—the presence of these glycosides in developed cell wall has not been reasonably demonstrated.) Since reduction of conjugated esters (**34** or **41**) was not easily accomplished, they first hydrogenated to produce the aliphatic esters (**35, 42**), and then borohydride reduced to the aliphatic alcohols **36, 43**. Free carboxylic acids **39** do not reduce with $LiBH_4$ and thus become distinguishable from the esters. From this scheme alone, which ultimately results

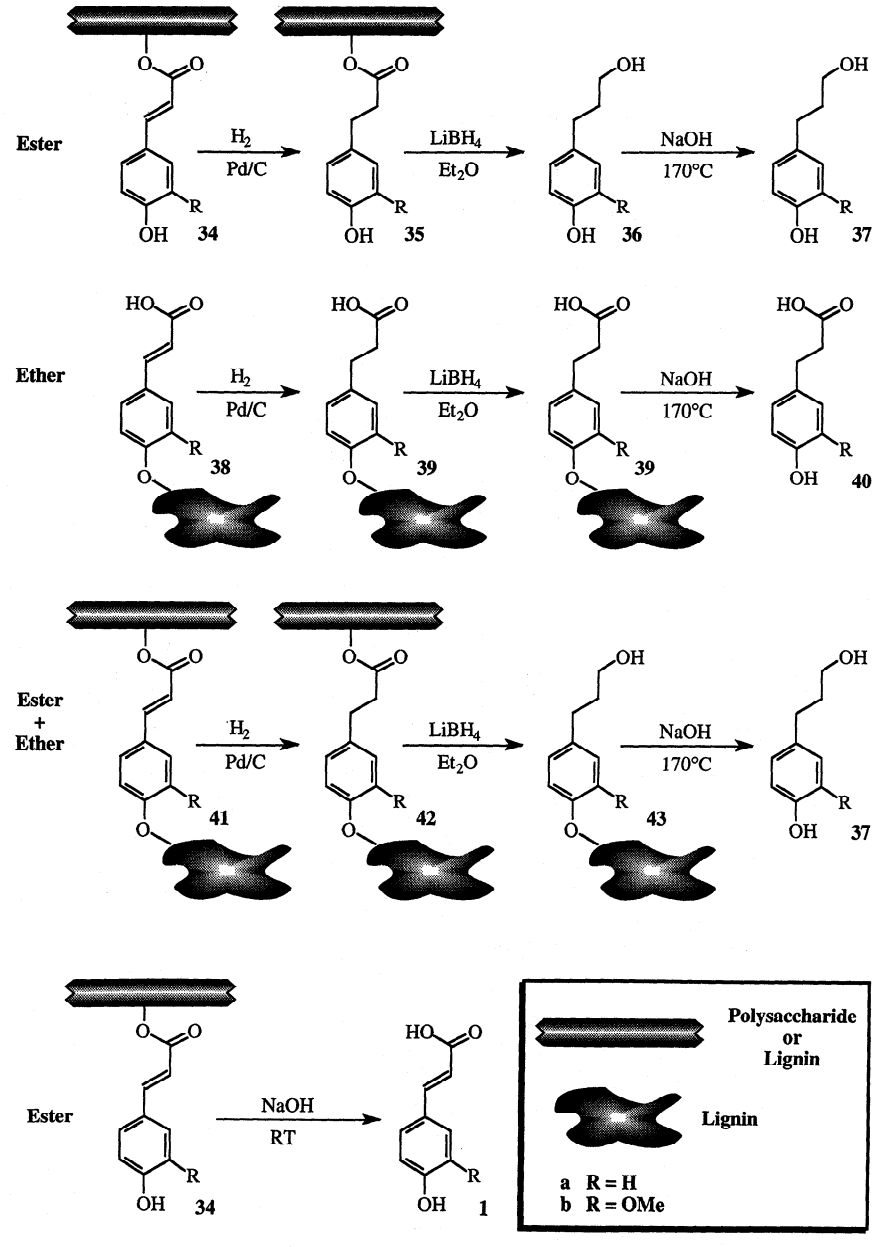


Fig. 9–8. Quantitative method for determining ester-linked, ether-linked, and both ester- and ether-linked hydroxycinnamic acids. Figure adapted from Fig. 1 of Lam et al., 1992.

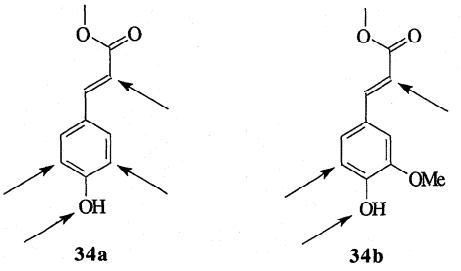
in monomers **37** and **40** (which can be quantitated by GC), it is not possible to distinguish between hydroxycinnamic acids that are esterified **34** vs. those that are both esterified and etherified **41**. However, a simple room temperature 1*M* NaOH hydrolysis (bottom scheme of Fig. 9–8) yields quantifiable

Table 9-2. Fraction (g/kg) of each type of hydroxycinnamic acid associated with cell wall fractions. Data from Lam et al. (1991b).

	p-Coumaric			Ferulic		
Sample	Ester	Ether	Bridged	Ester	Ether	Bridged
Wheat straw	0.113	0.031	0.000	0.069	0.000	0.141
90% Dioxane lignin	(78%)	(22%)	(0%)	(33%)	(0%)	(67%)
Phalaris	0.209	0.089	0.000	0.771	0.005	0.060
90% Dioxane lignin	(70%)	(30%)	(0%)	(52%)	(4%)	(44%)
Phalaris	0.038	0.007	0.000	0.031	0.000	0.051
50% Dioxane extract	(84%)	(16%)	(0%)	(38%)	(0%)	(62%)

hydroxycinnamic acids **1** resulting from the ester-only component **34**. The difference represents the quantity of hydroxycinnamic acids that are both ester and ether linked (in structures **41**). The revealing data for dioxane/water extracts of wheat and phalaris (*Phalaris aquatica* cd. Cirosa) internodes are shown in Table 9-2 (Lam et al., 1992). Clearly *p*-coumaric acid is heavily esterified to lignins (although there may be some contribution from carbohydrates in even the 90% dioxane/water samples) but also quite heavily involved in ether-bonding. Interestingly, *p*-coumaric acid does not appear to be involved in cross-linked structures. Ferulic acid, on the other hand, occurs esterified only, but the majority of it is both esterified and etherified. That essentially no ferulic acid is detectable in the ether-only form implies that it is pre-esterified ferulic acid that is incorporated into the lignin polymer. Whether this esterified ferulic acid is actively involved in the free-radical polymerization itself, or simply traps quinone methide polymerization intermediates, remains to be determined and is discussed later in this chapter.

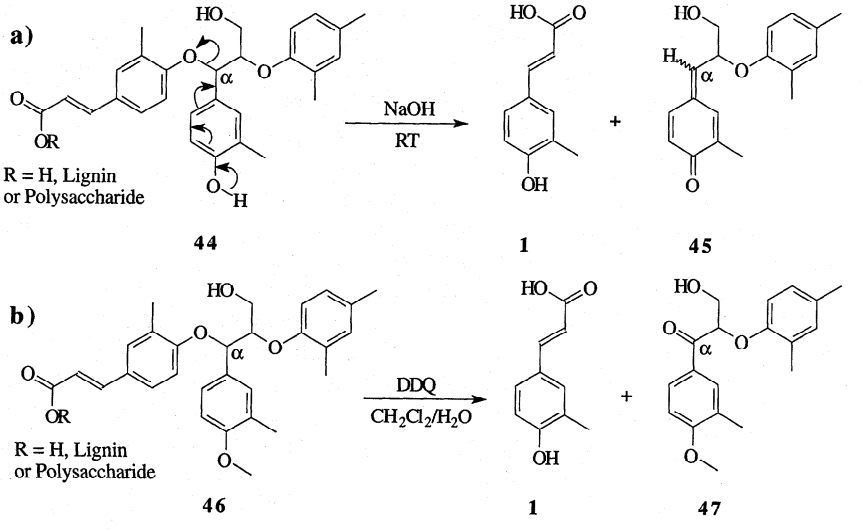
One complication in interpreting these data further is that they cannot reflect the possible contribution from condensed structures. It is well known that during lignification the aromatic ring 5-position in a guaiacyl unit is reactive and yields structures capable of forming branch-points in the lignin structure, namely the 5-*O*-4, 5-5 (biphenyl), and β-5 (phenylcoumaran) structures (Adler, 1977; Harkin, 1967, 1973). It is a widely held, although unsubstantiated, belief that condensation reactions are even more prevalent in *p*-hydroxyphenyl units which have the 3- and 5-positions available for reaction. If hydroxycinnamoyl esters are directly involved in free-radical coupling during lignification, Fig. 9-9 illustrates for a *p*-coumaryl ester **34a**, and a feruloyl ester **34b**, the sites at which condensation with other free radicals may occur—these coupling modes will be examined in more detail under section II.C.4 on Polymeric Models. What becomes clear is that none of these five-linked, β-linked, or condensed structures will produce compounds that analyze as being derived from hydroxycinnamic acids by any of the degradative schemes used today—base or acid hydrolysis, thioacidolysis (see chapter 6 by Lapierre in this book), or the method of Lam et al. (1991a,b). Consequently, the hydroxycinnamic acid contents of tissues reported may underestimate the actual amount of hydroxycinnamic acids that have been polymerized into the polymers. More importantly, while it may be tempting



34a 34b

Fig. 9-9. Possible sites for coupling reactions of radical 1-electron oxidized products of feruloyl esters **34a** and *p*-coumaroyl esters **34b**.

to conclude from Table 9-2 that *p*-coumaric acid is never involved in cross-linking, it may also be possible that all esterified *p*-coumaric acids incorporated into lignin undergo 5- or β-linking that makes them indeterminable by these methods. Another complication is usually overlooked, and its importance is unknown but suspected to be minor. That is that free-phenolic α-etherified structures **44** in lignin will cleave readily in 1*M* NaOH at room temperature (Fig. 9-10a) yielding phenolic acids **1** and lignin quinone methide structures **45**, and the phenolic acids **1** released this way become, incorrectly, identified as ester-linked structures. It is noteworthy that free-phenolic α-ester or α-ether model compounds, particularly of ferulic acid, are quite unstable, degrading over a period of months on storage (Helm & Ralph, 1992a,b). It is likely that units such as **44** (Fig. 9-10a) are unstable under physiological conditions also.



44 1 45

46 1 47

Fig. 9-10. (a) Mechanism for release of hydroxycinnamic esters or acids from free-phenolic α-ether structures. (b) DDQ oxidation proposed to liberate hydroxycinnamic acids from α-ether structures.

B. Regiochemistry of Lignin/Hydroxycinnamic Acid Interactions

The question of regiochemistry has been only tentatively explored. Shimada et al. (1971) claimed that *p*-coumaric acid was mainly esterified to the γ-position, based on the resistance of γ-esters to methanolysis, a method known to preferentially solvolyze the α-position. From acidolysis experiments on model compounds and bamboo milled wood lignin, Nakamura and Higuchi (1976) estimated the ratio of γ to α ester-linked *p*-coumaric acid to be about 80:20. However, ester-linked carbohydrates invariably associated with these lignins complicate the observations. Scalbert et al. (1986) have shown that, in organic solvents with base catalysis, hydroxycinnamic acids add cleanly to lignin model quinone methides giving α-esters, while hydroxycinnamoyl esters add through their phenolic group to give α-ethers. The addition of phenols under more physiological conditions has been more extensively studied by Brunow's group (Sipilä & Brunow, 1991; Sipilä, 1990). Watanabe et al. (1989, 1991) have an excellent method for determining α-linked carbohydrates (and determining the linkage position to the carbohydrate) that could potentially be used here to quantitate α-linked hydroxycinnamic acids (Fig. 9–10b). The method is based on DDQ (2,3-dichloro-5,6-dicyano-1,4-benzoquinone) oxidation, a reaction that is quite specific for the benzylic α-position. When coupled with methylation analysis of the released sugars, this method has been used to establish that glucouronoxylan in the tropical hardwood *Albizia falcata* was directly bound as an α-ether to the lignin through C-2 or C-3 positions of the xylose units. To our knowledge, this method has not been used to obtain additional regiochemical information on forages.

The location of alkali-extractable *p*-coumaric and ferulic acids in rice (*Oryza sativa* L.) has been studied by Terashima's group using autoradiographic techniques (He & Terashima, 1989, 1990). It was found that hydroxycinnamic acids were present in all types of cell walls and increased with the lignification process. Interestingly they also reported sinapic acid **1c** and suggested its connection to lignin. This group has done some of the most outstanding work examining morphological effects on lignin heterogeneity (e.g., He & Terashima, 1990), some of which is reviewed by Terashima et al. (see chapter 10 in this book).

Another class of structures with a potentially significant role in cross-linking the plant cell wall is the dimers of the hydroxycinnamic acids, both the 5-5 dimers (Hartley & Jones, 1978; Kamisaka et al., 1990; reviewed in: Yamamoto et al., 1989) and the [2+2]cyclo-addition dimers, the truxillic and truxinic acids (Hartley et al., 1988, 1990; Ford & Hartley, 1989, 1990; reviewed in: Yamamoto et al., 1989, Hartley & Ford, 1989; Monties, 1991). It has been shown that hydroxycinnamoyl esters on polysaccharides can dimerize photochemically to the truxillic esters (Hartley et al., 1990a). These products are found in plant cell walls and are presumably present in the growing plant. Since this process is photochemical, it is unclear how the plant is able to regulate the formation of such structures—they can only be formed if two hydroxycinnamic acids or esters are in sufficient proximity to bond.

If cross-linking of esterified hydroxycinnamic acids is to occur late in the development of the cell wall, the rigidity of the polymers implies that the probability of two hydroxycinnamoyl esters being close enough to bond is very low. During early development, this cross-linking between esters, or of the acids themselves, is more probable.

C. Model Studies

1. Lignin Models

Model compounds representing, to varying degrees, the variety of structural types in lignin, are commonly employed in lignin research. Well-chosen models with appropriate size and functionality to represent the structures of interest are invaluable in structural and reactivity studies.

The predominant free-radical condensation product in lignin biosynthesis leads to the *β-O*-4 linkage that accounts for 40 to 50% of inter-unit linkages (Sarkanen & Ludwig, 1971). Consequently, the *β-O*-4 dimers **48a**, Fig. 9–11,

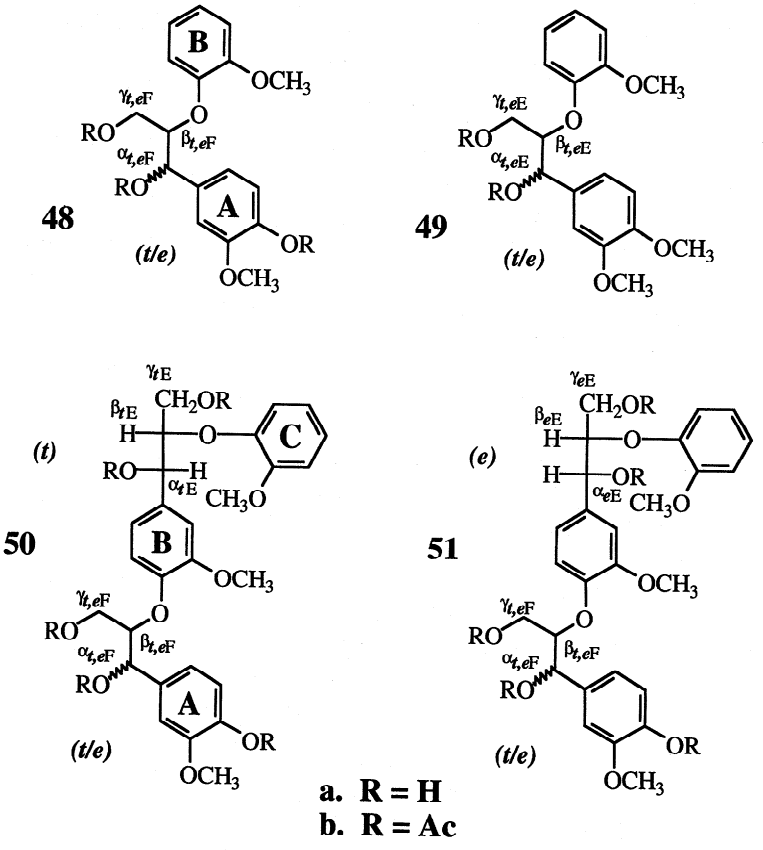


Fig. 9–11. Lignin model dimers guaiacylglycerol-β-guaiacyl ether **48**, veratrylglycerol-β-guaiacyl ether **49**, and lignin model trimers guaiacylglycerol-β-(guaiacylglycerol-β-guaiacylether)-ether **50** (the *t/e-t* isomers) and **51** (the *t/e-e* isomers).

have been extensively studied and there are now excellent synthetic routes available (Nakatsubo et al., 1975; Landucci et al., 1981; Ralph & Young, 1981). NMR assignments have been made unambiguously by using long-range heteronuclear correlation experiments and exploiting the long-range correlations with diagnostic OH protons (Ralph, 1988). A rapid proton NMR method for determining stereochemistry on small amounts of underivatized sample (Ralph & Helm, 1991) has recently become available to improve the older NMR methods that required derivatization (acetylation and $\geq$200 MHz proton NMR [Nakatsubo et al., 1975; Ralph, 1982a,b], trifluoroacetylation and ^{19}F or $\geq$200 MHz proton NMR [Ralph & Wilkins, 1985] or larger quantities of substrate and excessive instrumental time [quantitative ^{13}C NMR]). New gas and liquid chromatographic methods have also been recently developed (J. Ralph, S. Quideau, I.D. Suckling, and R.M. Ede, 1990, unpublished data).

The use of trimeric lignin model compounds is a natural extension toward more complex and accurate modeling of lignin for structural and reactivity studies (Katayama et al., 1987; Kamaya et al., 1980; Namba et al., 1980; Ralph et al., 1986; Nakatsubo & Higuchi, 1980a,b; Hyatt, 1987; Brunow et al., 1989). In the NMR spectra of dimers such as guaiacylglycerol-β-guaiacyl ether (GGβGE) **48a**, it has long been recognized that, while the modeling of sidechain and free-phenolic end of the molecule is quite accurate and gives chemical shifts that are in good agreement with higher oligomers and the lignin polymer itself, the B-ring is only poorly modeled since it possesses no sidechain. In particular the carbons of this ring, although they have been unambiguously assigned (Ralph, 1988), are of little value for the case of true lignins. Etherified units, internal to the lignin structure, are often simply modeled by using methylated (veratryl) analogues **49**, and this too is an imperfect model. Trimeric compounds **50**, **51** allow accurate modeling of the free-phenolic end and the internal unit, although the trimer C-ring suffers from the same inadequacies as the B-ring in dimers (Ralph & Rodger, 1991). But such trimers do allow NMR parameters to be accurately determined for both the free-phenolic and the internal units in lignin chains.

GGβGE **48a** has two optical centers, allowing four optical isomers, and is synthesized generally as a mixture of *threo* and *erythro* forms. There are now methods available to synthesize predominantly the *threo* or *erythro* isomer, and they are relatively easy to obtain in pure crystalline form (Ralph & Helm, 1991; Ralph & Young, 1981; Nakatsubo et al., 1975; Brunow et al., 1988). For the trimeric models **50**, **51** (Fig. 9–11), however, it is soon apparent that there are 16 (2^4) possible optical isomers due to the four optical centers (Ralph & Rodger, 1991). The number of distinct (non-optical) compounds is 8 ($2^4/2$ or 2^{4-1}). This number of possibilities can be halved by constraining the dimeric compound used in the linear synthesis to be either the *threo* **50** or *erythro* **51** form (Ralph et al., 1986). The other sidechain was produced quite nonselectively via ketone reduction and was of mixed *threo/erythro* stereochemistry, although methods exist for obtaining about 80% *threo* selectivity in this reduction step (Ralph & Helm, 1991; Brunow et al., 1988; Ralph & Young, 1981; Hosoya et al., 1980). It is tempting to

think that fixing the stereochemistry of one unit of the trimer will leave just two isomeric possibilities; for example, if the fixed unit is *threo*, both *threo-threo* and *erythro-threo* possibilities seem logical. However, this represents only one-half of the possibilities since fixing the second unit to *threo* constrains only the relative stereochemistry of the two optical centers—they could still be *RR* or *SS*. Consequently, there are still eight possible optical products or four physical isomers. Fixing the stereochemistry of the second unit has halved the number of possibilities.

There are two basic approaches to trimeric models, and onward to higher oligomers. The first is the simple, nonconvergent, synthesis involving nucleophilic addition of preformed dimers to an α-bromoketone (Ralph et al., 1986). A particular advantage is the ability to use stereochemically pure dimers thus reducing the number of isomeric possibilities, as noted above. The only drawback in this approach is the difficulty of hydroxyformylation of the keto intermediates. Convergent syntheses based on Nakatsubo's earlier dimer syntheses have also been successful (Katayama et al., 1987; Kamaya et al., 1980; Namba et al., 1980; Nakatsubo & Higuchi, 1980a,b; Hyatt, 1987, Brunow et al., 1989) and recent improvements have been made in both yield and isomer selectivity (Ragauskas, 1991a,b). Brunow et al. (1989) have dimerized these trimers via 5-5 coupling of the terminal phenolic end to form hexamers.

The trimers, such as the β-ether/β-ether trimers **50a/51a** and their acetylated derivatives **50b/51b**, have been a source of spectacular NMR spectra and provide a novel insight into the isomeric complexity of the lignin polymer that seems to have escaped previous attention. Figure 9–12 shows selected regions of the 600 MHz 1D spectra of the acetates of guaiacylglycerol-β-guaiacyl ether **48b**, veratrylglycerol-β-guaiacyl ether **49b**, the *t/e-t* trimer **50b** and the *t/e-e* trimer **51b**. Further discussion of these spectra is worthwhile and is facilitated by use of the following conventions. In describing a proton, the first letter or letter pair refers to the atom, that is, α, β, γ_1, or γ_2 for each of the four protons on the sidechain. The second term, subscripted, italic and bold, refers to the isomer, ether *t* or *e* for *threo* or *erythro*, and the final term, a capital letter, plain, also subscripted, refers to the position of the unit within the structure, E for etherified (internal unit), F for free (terminal free-phenolic end). Thus $\alpha_{\boldsymbol{e}\mathrm{F}}$ refers to an α-proton, in a unit with *erythro* stereochemistry, at the terminal or free-phenolic end of the molecule.

It is the α-proton resonances of acetylated models that are often used to determine stereochemistry and quantitate *erythro:threo* ratios (Ralph & Young, 1981; Ralph & Wilkins, 1985). They are well dispersed in the dimers, for both etherified **49b** and free-phenolic **48b** models, even at relatively moderate field strengths (200 MHz). In the α-proton region of trimer **51b**, the etherified proton resonances ($\alpha_{\boldsymbol{e}\mathrm{E}}$) are well dispersed from the free-phenolic ones, and the free-phenolic *threo* ($\alpha_{\boldsymbol{t}\mathrm{F}}$) and *erythro* ($\alpha_{\boldsymbol{e}\mathrm{F}}$) resonances are also well dispersed. Clearly, each of the free-end unit α-protons show both possible sets of resonances. For example, the pair of $\alpha_{\boldsymbol{t}\mathrm{F}}$ doublets arise from a *threo* (*RR/SS*) A/B moiety and an *erythro* (*RS/SR*) B/C moiety resulting in four

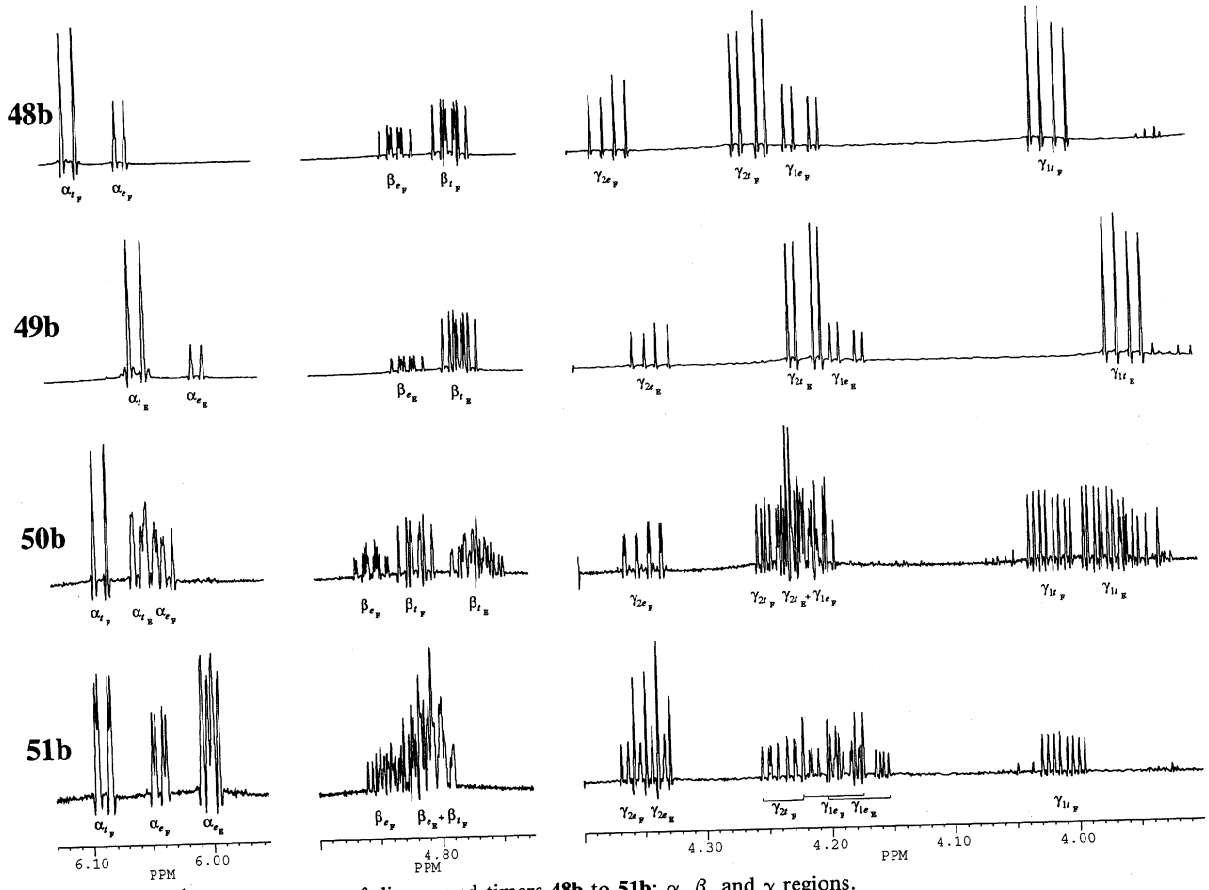


Fig. 9-12. Partial 600 MHz ^{1}H NMR spectra of dimers and timers **48b** to **51b**; α, β, and γ regions.

optical isomers (*RR-RS, RR-SR, SS-RS, SS-SR*) and two physical stereoisomers. In the etherified unit (peaks α_{eE}), all four isomers (from an *erythro* B/C moiety plus an either *erythro* or *threo* A/B moiety) are distinguishable. A recent inverse-detected C-H correlation (Bax & Subramanian, 1986) experiment (not shown) confirms the assignments, since the *threo* and *erythro* isomers have well-known and different chemical shifts, whether etherified or free (Ralph, 1982a,b). The α-region of trimer **50b** (the *t/e-t* compound) is quite different. Here, all four isomers have magnetically indistinct *threo* free-end resonances and a single doublet is observed (peak α_{tF}), but the α_{eF} peaks are disperse and overlap with the etherified peaks, α_{tE}.

The β-region is informative. The β-protons in dimers **48b** and **49b** are well resolved at 600 MHz, and in trimer **50b**, all three β-proton possibilities (β_{eF}, β_{tF}, and β_{tE}) are readily assigned. The internal etherified unit β_{tE} displays more dispersion among the isomers than the free-end protons. In trimer **51b**, the dispersion of the β-proton classes is less, and β_{eE} and β_{tF} overlap severely.

The γ region is perhaps the most amazing. In the dimers **48b** and **49b**, each isomeric proton is well resolved. There are four γ-protons in each trimer, two on each unit. The *threo*-γ_1 protons in both the dimers and trimers are well resolved at high field (low δ) as are the *erythro*-γ_2 protons that resonate downfield from the other γ-protons, Fig. 9–12. In the *t/e-t* trimer **50b**, the γ_{2eF} protons are resolved into two magnetically different pairs, as are the γ_{1tF} protons. But it is the γ_{1tE} protons that clearly show all 16 peaks from the dd's of each of the four isomers. This is more clearly illustrated in Fig. 9–13, an expansion of this region. No attempt has been made to further refine the assignment of these four isomeric protons. The spectrum of trimer **51b** exhibits a γ_{1tF} peak cluster that is fully resolved at high field. The γ_{2eF}, and γ_{2eE} peaks overlap, but are fully assignable, the large dd clearly being due to the magnetic equivalence of all four isomers of γ_{2eE}, and the two smaller dd's from the two pairs of magnetically equivalent γ_{2eF} peaks. This assignment is corroborated by the single set of four intense contours and

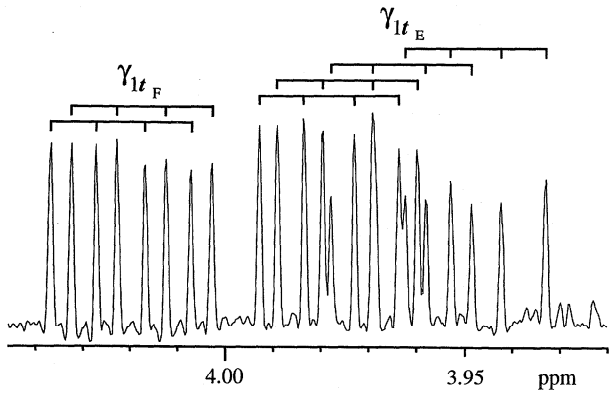


Fig. 9–13. Expansion of part of the γ-proton region of **50b**, showing dd's for each of the four isomers of the γ_{1tE} proton.

the two sets of four less intense contours in the J-resolved spectrum of Fig. 9–14. Two distinct γ_{2tF} dd's are also just resolved from the more complex and overlapping γ_{1eF} and γ_{1eE} protons, whose assignments are confirmed from the COSYRCT2 spectra (below). The 500 MHz J-resolved spectrum of Fig. 9–14 (deliberately not tilted or symmetrized so that the peak positions can be correlated) clearly shows the dd nature of each resonance.

The COSY and long-range COSY spectra of these compounds are also informative, especially for assigning some of the aromatic protons, but it is experiments that are capable of correlating all protons within a spin system that are the most valuable. These experiments define those protons belonging to each of the four isomers for each trimer and allow unambiguous assignments because it is possible to make at least one unequivocal sidechain proton assignment. The prime experiment of this type today is the HOHAHA or TOCSY experiment (Braunschweiler & Ernst, 1983; Davis & Bax, 1985), but older instruments are often not capable of performing them. A less efficient, but remarkably useful and surprisingly flexible, alternative is the relayed coherence transfer experiment (Bax & Drobny, 1985). In this experiment, magnetization is passed sequentially from one proton in a molecule to an adjacent, coupled proton. The pulse-sequence delays are chosen to maximize this transfer, and the spectra are often completely detailed before the full complement of increments is taken. In fact, the spectrum of Fig. 9–15 results from processing only the first 200 of 512 increments because the intensity of desirable cross peaks is greater in the early part of the t_1 domain.

The COSYRCT2 spectrum of the *t/e-e* trimer **51b**, Fig. 9–15, is particularly easy to analyze because there is a single band of contours from the well dispersed α-protons that give correlations with their respective β and γ protons. Thus, for example, it can easily be seen from Fig. 9–15 that the β resonances to the low field (high δ) end belong to the *erythro* isomer of the free-phenolic unit of the trimer. Similarly, the very high field γ resonances belong to the *threo* isomer of the free-phenolic end unit. The correlations obtained with this experiment confirm the assignments noted on Fig. 9–12.

At this point, a brief diversion to discuss crystallinity in the lignin/hydroxycinnammic acid complex is worthwhile. The question of crystallinity or ordered regions in lignins is occasionally raised (Goring, 1989). It may be reasonable to expect some degree of order if lignin polymerization is affected by the regular carbohydrates that may be present as templates (Atalla & Agarwal, 1985). However, it becomes obvious from studying relatively simple trimeric compounds (above) that there is a great deal of overlooked complexity in lignin structures. For each C_6–C_3 unit that is added to a structure, two more optical centers are added, and four times as many physical isomers become possible. What does this mean for polymeric lignin? Is the occurrence of stereochemically ordered regions likely, and can we even expect to crystallize small lignin fragments?

It should be clear already that the number of isomer possibilities is likely to become huge for a molecule of mild complexity. It was recently illustrated (Ralph & Rodger, 1991) that a regular β-ether polymer (with no other structural entities involved) with a DP of just 110 (molecular weight

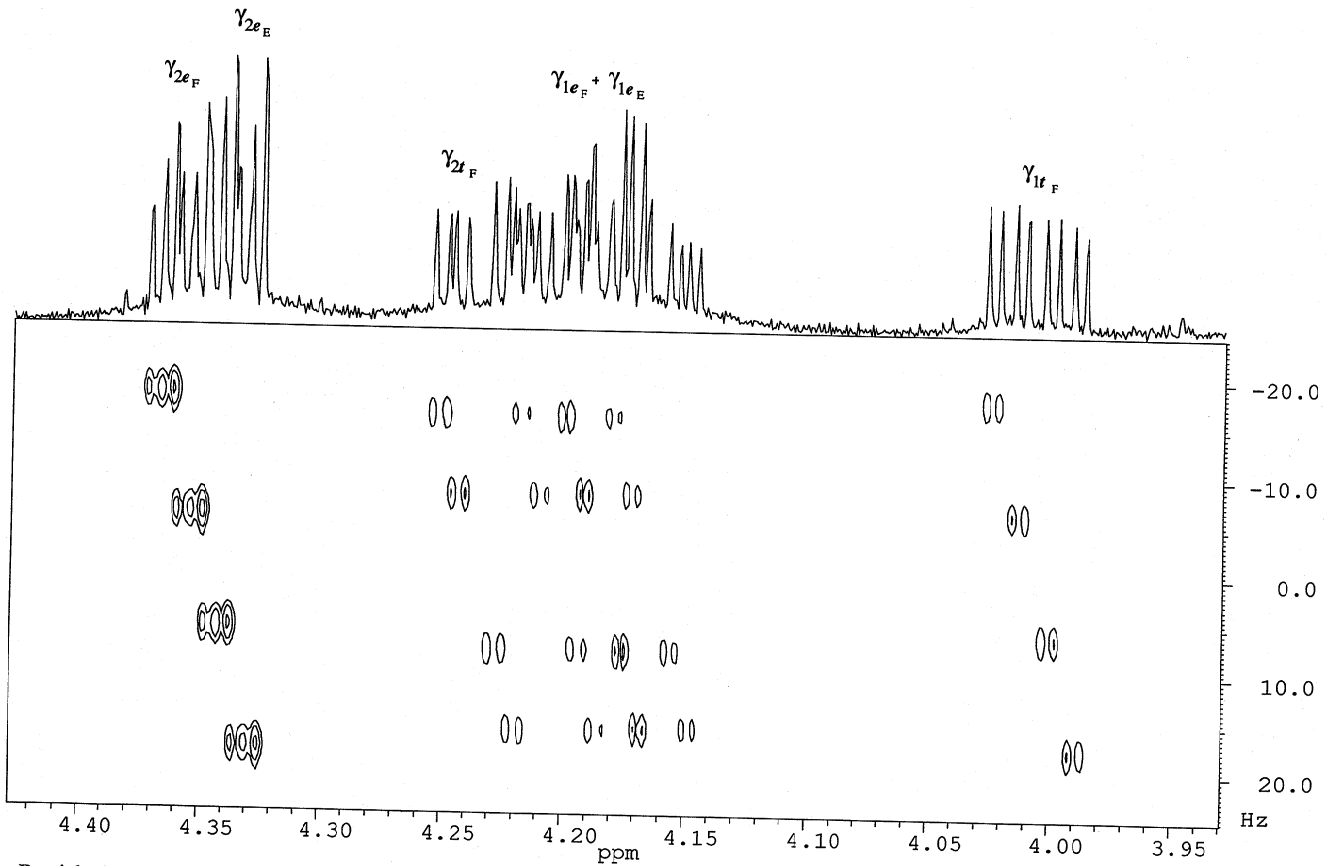


Fig. 9-14. Partial 500 MHz J-resolved spectrum of **51b** showing the dd nature of the γ-protons.

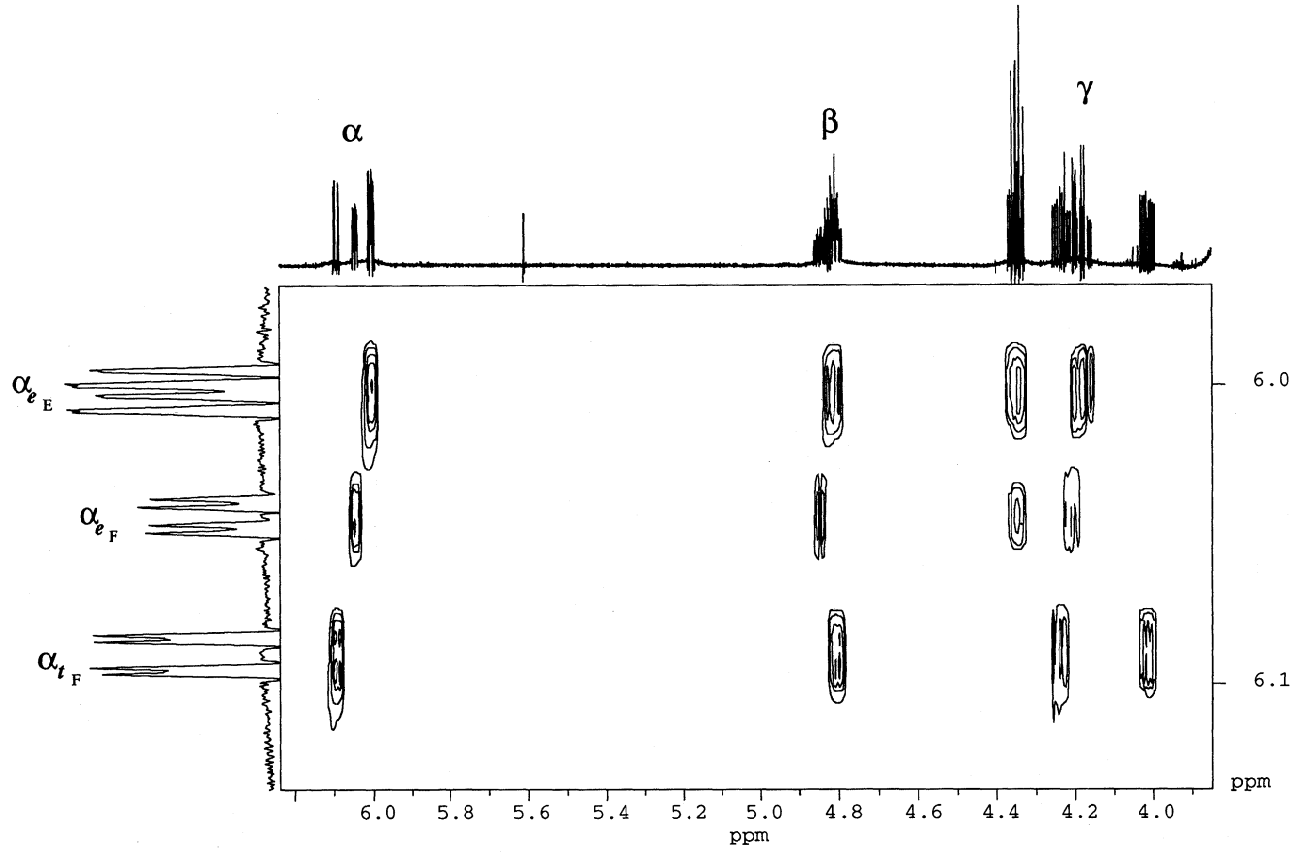


Fig. 9-15. Partial 600 MHz COSYRCT2 spectrum of **51b** showing the correlation of each α proton with each β and γ proton in the same isomer (same coupling network).

about 21 500) would have 10^{66} isomeric possibilities (there are about 10^{66} atoms in the galaxy!), and to isolate just 1 mg of a single isomer (which might be crystalline) would require approximately twice the weight of the earth of an even smaller sterically random β-ether 47-mer. Unless there is some evidence that there are biological templates for forming regular and isomerically identical chains of units into a lignin polymer, the idea of crystallinity, even just crystalline regions, in lignin must be seen as astronomically improbable.

2. Lignin-Hydroxycinnamoyl Ester Models

Three hydroxycinnamoyl ester derivatives of the β-aryl ether dimer lignin model **48a** are possible, **52-54** (Fig. 9–16). It should be noted that both the *threo* and *erythro* isomers of these structures, in the synthesized models and in the lignin structure itself, are likely to occur. Since NMR data is sensitive to isomeric form, each isomer needs to be fully characterized. Models **52-54** represent the ester functionality in the lignin polymer, but it should be clear that structures involving non-β-ether units (such as phenylcoumarans, biphenyls, β-C-1's) are also possible. It should be noted that each ester depicted represents a different biochemical pathway.

The α-ester **52** can be formed without the aid of enzymatic catalysis if the acid is present during lignification. Trapping of the intermediate quinone methides by such acids is well documented in model systems (Nakatsubo, 1981; Scalbert et al., 1986) and is plausible in the polymeric system.

We consider the phenolic ester **54** to be an unlikely structure since it would require that esterification occur after lignification. Since esterification of phenols is not a reaction that occurs readily at ambient temperatures under physiological conditions, the formation of such esters would require activation of the acid (e.g., as an S-CoA derivative) and presumably require the assistance of a thiol-transferase. This enzyme would have to act on the polymeric material, not on monomers, and no evidence for this type of reaction or structure has been noted.

The γ-ester **53** cannot be formed during the lignification step either. The likely presence of this structure (particularly with *p*-coumaric acid) in the

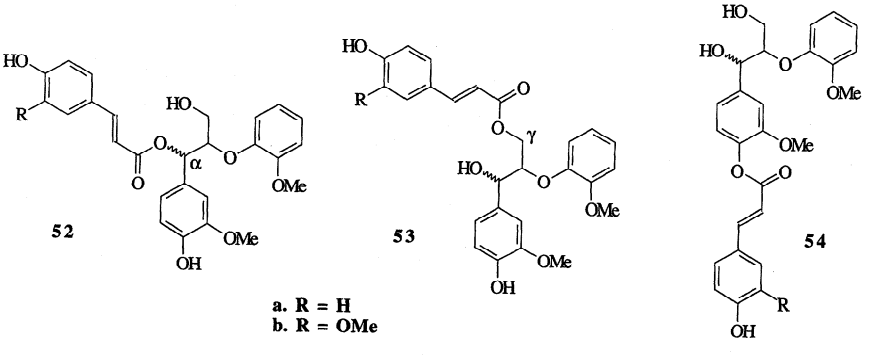


Fig. 9–16. The three possible mono-hydroxycinnamoyl ester derivatives of guaiacylglycerol-β-guaiacyl ether **48a**, the α-ester **52**, the γ-ester **53**, and the phenolic ester **54**.

lignin/hydroxycinnamic acid complex presumably stems from esterification of the cinnamyl alcohol monomers in the cytoplasm. These esterified cinnamyl alcohols would then polymerize into the lignin structure keeping the ester link intact. The method of this polymerization and the possible structures for the γ-esterified structures will be presented in section II.C.4 on Polymeric Models.

Synthesis of lignin/hydroxycinnamoyl ester model compounds **52-54** has been accomplished as outlined in Fig. 9–17. Simple protection/deprotection strategies were employed where necessary. The phenolic and the γ-esters were available from direct esterification of guaiacylglycerol-β-guaiacyl ether **48a**. The α-ester was generated from addition to the quinone methide **57**, readily generated from **48a** (Ralph & Young, 1983). Authenticated NMR data for both isomers of all these compounds are reported elsewhere (Helm & Ralph, 1992b).

3. Lignin-Hydroxycinnamyl Ether Models

Only two etherified structures based on the β-ether model are possible, **55** and **56** (Fig. 9–18). One is frequently overlooked and yet it is not only plausible but invokes entirely different biochemical implications. The delineation of the regiochemistry of phenolic acid ethers on lignin structures remains critical and will give additional insights into the biochemical pathways that may be manipulated for gains in cell wall digestibility.

The α-ether structure **55** can result in an analogous way to the α-ester structure **52**, namely simple nucleophilic addition to quinone methide intermediates (Scalbert et al., 1986). However, since an acid group is more likely to add than a phenol, it is necessary that the hydroxycinnamic acid be already esterified. Esterification to lignin structures (e.g., as a γ-ester) or to carbohydrates (e.g., C-5 of α-L-arabinofuranosyl units in arabinoxylans) has been described above. The implications of this structure are simply that the esterified hydroxycinnamic acids, retaining their phenolic groups free, are present in the matrix during active lignification and are little more than opportunistic nucleophiles available to trap the quinone methide intermediates from traditional lignin polymerization.

It is generally thought that attack on the quinone methide is the mechanism by which feruloyl esters become bound to lignin. This is a reasonable and predictable occurrence, the validity of which can be demonstrated in model systems (Scalbert et al., 1986). It has several frequently overlooked shortcomings however. Firstly, the feruloyl ester has to compete for the quinone methide with other nucleophiles including other phenols (from cinnamyl alcohol monomers and from the growing lignin polymer itself), acids present in other cell wall components (e.g., uronic acids), and water. In isolated lignins, at least 90% of the β-ether quinone methides formed result in α-hydroxy structures indicating that water is the primary addition product (Adler, 1977; Harkin, 1967, 1973). In cases where acids are in competition with phenols, esters are produced almost exclusively (R.F. Helm and J. Ralph, 1990, unpublished data; Scalbert et al., 1986; Nakatsubo, 1981). A more philosophical question arises when feruloyl esters and lignification are considered in

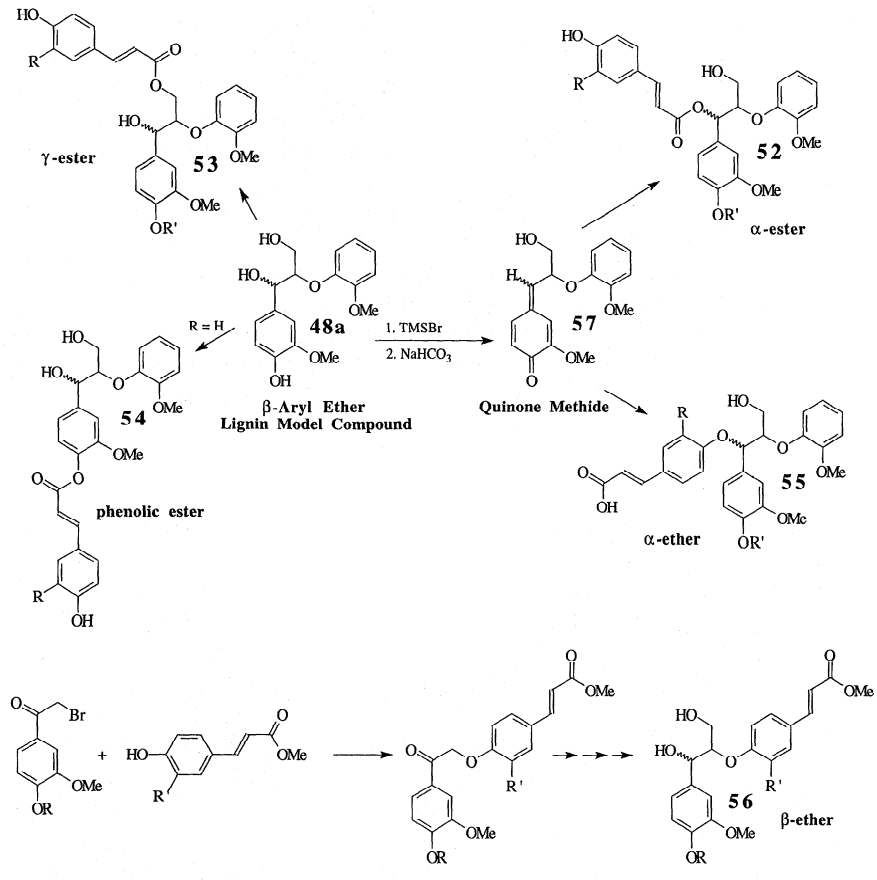
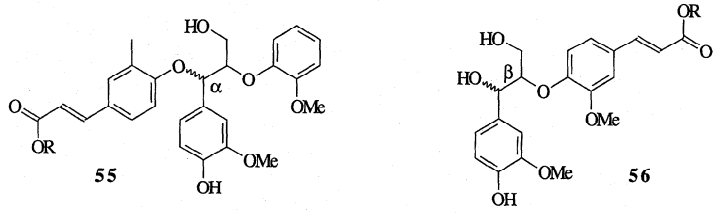


Fig. 9-17. Synthesis of model lignin/hydroxycinnamic acid compounds. Lower scheme: Synthetic scheme for β-hydroxycinnamyl ether models.

relation to the development of the plant cell wall. Feruloylated polysaccharides, typically arabinoxylans, are theorized to cross-link to lignin to impart various properties to the plant cell wall (as described in the Introduction of this chapter). Thus, it seems rather unlikely that the method of cross-link



a. R = H
b. R = Me
c. R = Ara-Me

Fig. 9-18. The two possible mono-hydroxycinnamyl ether products related to guaiacylglycerol-β-guaiacyl ether **48a**, the α-ether **55**, and the β-ether **56**.

formation would involve an uncontrollable reaction, especially when considering the spatial problem of placing a quinone methide sufficiently close to the feruloylated polysaccharide to produce the α-ether. Finally, although some peroxidase specificity has been demonstrated, it seems unlikely that these phenolic feruloyl esters would be available in the matrix for addition to quinone methides and yet not be amenable to H-abstraction and the radical coupling process. Consequently, we suspect that feruloyl ethers are also incorporated into the lignin structure through co-polymerization with lignin monomers.

The seldom-mentioned β-ether **56** has far more interesting implications. This structure absolutely requires that the hydroxycinnamic acid, presumably again in an already esterified form, is directly involved in the peroxidase-assisted free-radical polymerization. Finding β-hydroxycinnamic acid structures in the lignin/hydroxycinnamic acid complex would lend support to the theory that feruloylated arabinoxylan may act as a template for lignification (Fry, 1985; Yamamoto et al., 1989), the lignin polymer growing from the surface of the xylan fraction. As plants produce significant numbers of peroxidase isoenzymes, specific peroxidases for H-abstraction from these more conjugationally extended phenols cannot be ruled out. This would allow the plant some regulation of an otherwise rather uncontrolled process.

The synthesis of the α-ether model **55**, via the quinone methide **57**, is shown in Fig. 9–17. Synthesis of the β-ether model **56** was via an analogous scheme to the synthesis of β-ether lignin models described above, as outlined in the lower scheme of Fig. 9–17. Fully authenticated NMR data from these compounds are reported elsewhere (Helm & Ralph, 1992a).

4. Polymeric Models

Plant cell wall lignins are synthesized by the polymerization of radicals generated from one-electron oxidation of the hydroxycinnamyl alcohols **2** (Adler, 1977; Harkin, 1967, 1973). Whereas most gymnosperm lignins are derived solely from coniferyl alcohol **2b**, herbaceous plant lignins derive principally from two monomers, coniferyl alcohol **2b** and sinapyl alcohol **2c**, with small amounts of *p*-coumaryl alcohol **2a** also being involved. Cell wall lignin is generally regarded to be an endwise polymerization process rather than a bulk process, with new monomer radicals coupling with radicals generated in the growing polymer (Kirk & Brunow, 1988). As determined by Freudenberg and Erdtman during early discoveries on this enigmatic polymer (Reviewed in Adler, 1977; Harkin, 1967, 1973; Nakatsubo, 1981), this radical coupling process can be mimicked in vitro to produce synthetic dehydrogenation polymers or DHPs. DHPs have been used in a wide variety of studies although have sometimes drawn criticism because they have been used inappropriately. It must be recognized that they can differ substantially from native lignins (Lewis et al., 1987; Brunow & Wallin, 1981; Gagnaire & Robert, 1978; Nimz et al., 1974, 1981; Faix & Beinhoff, 1988; Faix, 1986; Robert & Brunow, 1984; Brunow & Lundquist, 1991), although certain critical enhancements to the preparation methods have made vast improvements. These

include: the enzymatic generation of hydrogen peroxide in situ (Lyr, 1957; Tollier et al., 1991), pH considerations (Sipilä, 1990), and novel polymerization techniques (Tanahashi et al., 1981, 1982). Additionally, since they are usually prepared free of the remaining cell wall matrix, they can lose the important cross-linking and matrix effects that are important in the reactivity of the intact cell wall.

In studies of the attachment and regiochemistry of hydroxycinnamic acids into the lignin/hydroxycinnamic acid matrix, the use of synthetic DHPs can be of enormous mechanistic value. Using DHPs it becomes possible to unambiguously determine what types of products can be formed during this critical free-radical polymerization step. By incorporating hydroxycinnamic monomers, particularly with strategic ^{13}C labels, it becomes possible to delineate the actual linkages available to hydroxycinnamic acids from the myriad of structural types possible. While these studies indicate structures that are chemically possible, it will remain to check the plant system for each of these possibilities since the presence of cell wall components can affect reaction pathways. This has most recently been demonstrated by the observations reviewed by Fry and Miller (1989) on the peroxidase assisted oxidative coupling of tyrosine—all in vitro studies formed predominantly di-tyrosine, the 3-3 linked structure, whereas isodityrosine (3-*O*-4) was the exclusive product in vivo.

To determine which coupling modes are available for hydroxycinnamic acids and their esters, DHP's can be prepared with hydroxycinnamic models added to the lignin monomer (Shimada et al., 1971; Nakamura & Higuchi, 1978a,b). In the remainder of this section we shall, for simplicity reasons, restrict our attention to examining only those units arising from coniferyl alcohol, i.e., guaiacyl units. Additionally, we will not examine all the radical coupling modes, but just those that lead to lignin's predominant structures, the β-aryl ether (β-*O*-4), phenylcoumaran (β-5), diaryl ether (5-*O*-4), and biphenyl (5-5) structures. These structures in native lignin arise from coupling of radicals that are conveniently written as resonance forms **59x**, **59y**, and **59z**, Fig. 9–19. (It must be emphasized that these simply represent three of the five alternative localized resonance forms of the same radical—they are not distinct entites!).

Incorporation of FA-Ara **33b**, the 5-*O*-feruloyl ester of methyl α-L-arabinofuranoside, into polymerizing lignin will be examined first. As noted above, the esterified ferulic acid may either add to the quinone methide to form α-ethers, or it may actively participate in the free-radical polymerization. It is the latter case that gives rise to a plethora of bonding possibilities as outlined in Fig. 9–19 and 9–20. Figure 9–19 shows the initial reactions that are possible from the feruloyl ester radical **58** and the coniferyl alcohol radical **59**. For simplicity, the controversial β-C-1 structures (involving reactions with resonance forms with the free electron at the 1-position) are not considered in this analysis. Combining radical **58**, with radical **59**, can lead to eight possible dimeric products as shown in Fig. 9–19. The products shown here are not the intermediates, but the assumed final products. In the 4-*O*-β' model **60a/z** [the naming convention specifies the FA-Ara coupling site

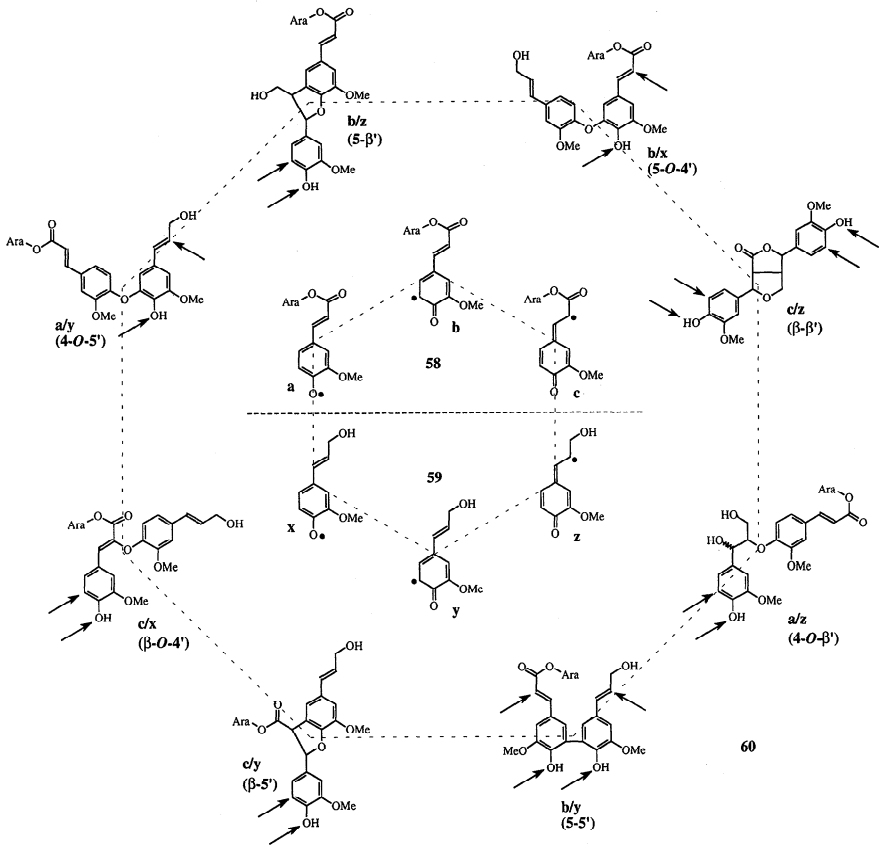


Fig. 9–19. Coupling of the radical **58** from FA-Ara with the coniferyl alcohol radical **59**. Only three of the five resonance forms of the radical are considered, giving rise to eight possible dimeric coupling products **60**. The sites where further radical polymerization can occur are arrowed. The convention used for describing adduct compounds **60** is illustrated with an example: **60a/z** represents the final product resulting from coupling of the formal radicals **58a** and **59z**, the FA-Ara radical being specified first; the descriptive nomenclature, e.g., *β-O-4'*, follows standard lignin conventions with the first term indicating the coupling site of FA-Ara and the second (primed) site to the coniferyl alcohol or lignin moiety.

first and the coniferyl alcohol or lignin moiety coupling site second (primed)—see legend to Fig. 9–19], the α-position is shown substituted with an hydroxyl. While this is the most prevalent α-substituent, it could alternatively be an ether (to an already growing lignin polymer, a monomer, or an esterified hydroxycinnamic acid), or to an hydroxycinnamoyl ester or uronic acid moiety on a polysaccharide. Adduct **60c/z** is the assumed lactone resulting from β-β' coupling. It should be noted that this product is unique in having eliminated the arabinosyl component. The proposed mechanism is shown later in Fig. 9–25. Structures **60c/y** and **60c/z** differ from the others in that they involve destruction of the conjugated double bond in the feruloyl-derived moiety. This arises, in each case and for **60c/x**, from coupling to the β-position of **58**. It was not previously known whether this coupling mode

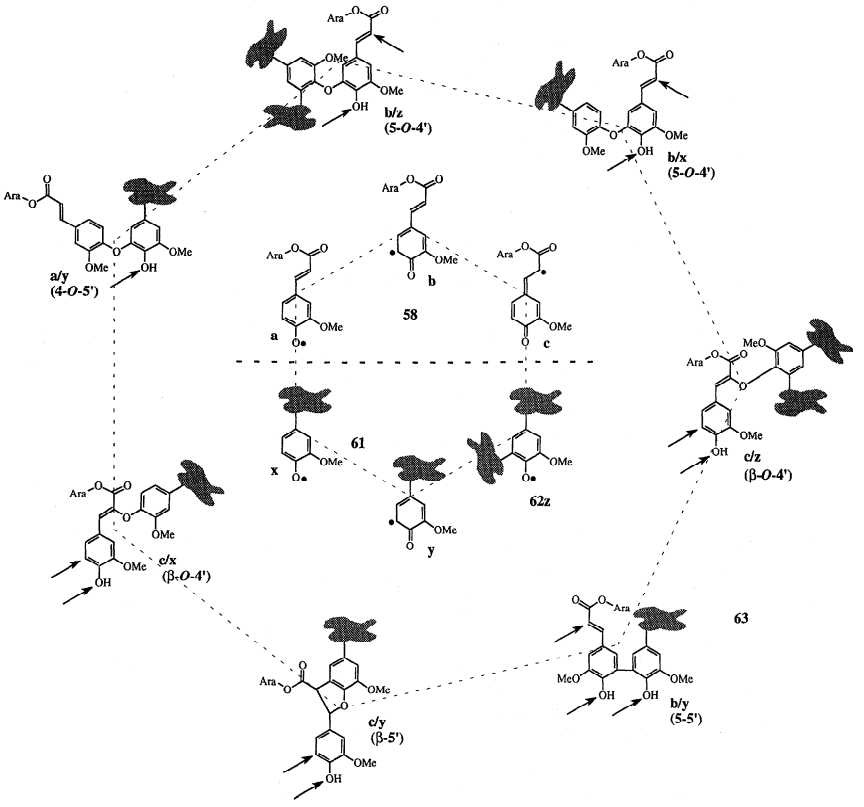


Fig. 9–20. Coupling of the radical from FA-Ara **58** with radicals from a growing lignin polymer (guaiacyl radical **61**, and five-substituted guaiacyl radical **62**). Only three of the five resonance forms of radical **58** and only three possible lignin radicals are considered, giving rise to seven possible coupling products **63**. The sites where further radical polymerization can occur are arrowed. The convention for naming products **63** is analogous to that used for **60** in Fig. 9–19.

is more or less important for the more conjugated feruloyl ester than for coniferyl alcohol where β-linked structures, phenylcoumarans (β-5) and pinoresinols (β-β), can account for about 9 to 15% of the inter-unit linkage types (Adler, 1977). As indicated by the arrows in Fig. 9–19, these dimeric units have a variety of ways of polymerizing further into the lignin/hydroxycinnamic acid polymer. Figure 9–20 is an analogous scheme depicting the reactions that are possible for incorporating FA-Ara **33b** into an already growing lignin polymer. Again to restrict the number of possible structures for consideration, only those involving 4- and 5-bonding are considered—structures maintaining the unsaturated α-β bond are capable of many more possibilities. Radical **62** represents 5-linked structures and any reactions involving these units create branch-points in the evolving polymer. Again the β-coupling of radical **58c** produce products in which the sidechain becomes fully saturated and the ester function is unconjugated.

One factor very evident from Fig. 9–19 and 9–20 is that, if these reactions also occur in vivo, the amount of hydroxycinnamic acids involved in the lignin/hydroxycinnamic acid complex may well be higher than supposed. Only some of the units would cleave under typical acid- or base-hydrolysis conditions to yield the hydroxycinnamic acid. These are represented by dimers **60a/y** and **60a/z** in Fig. 9–19, and **63a/y** in Fig. 9–20. All of the other structures either contain unhydrolyzable C-C bonds, or have saturated sidechains that masks their origin as being from hydroxycinnamic acids. *p*-Coumaric acid may be expected to undergo even more extensive 5,5′, 5-*O*-4′, or 5-*β*′ coupling with other lignin moieties and would become equally inaccessible to the hydroxycinnamic acid determination procedures. This is a further reason why the conceptual separation of phenolic components from lignin in forage plants is a dangerous concept. Current analytical techniques simply cannot ascertain which moieties in the lignin/hydroxycinnamic acid complex arise from hydroxycinnamyl alcohols (the traditional lignin precursors) and which arise from cross-linking reactions of hydroxycinnamic acids.

The FA-Ara DHP **64**, Fig. 9–21, was prepared according to Kirk and Brunow's (1988) Zutropf-like method by separate dropwise addition of two buffered solutions (pH 6.5), one containing the FA-Ara **33b** (40% γ-13C-labeled) and coniferyl alcohol **2b** monomers (1:9) and the peroxidase enzyme, and the other containing hydrogen peroxide (H_2O_2), to a stirred volume of pH 6.5 buffer, in the dark at ambient temperature (Ralph et al., 1992a). The level of **64** was chosen at 10% to minimize statistical homocondensation products, while giving a sufficent loading to determine products.

Long-range C-H correlation NMR experiments are particularly valuable for establishing connectivity. Figure 9–22 shows a small subsection, incorporating only the carbonyl carbon region, of an ^{1}H-detected long-range 2D C-H correlation spectrum of DHP **64**, and the corresponding region of several dimers synthesized for proof of the assignments made. It is immediately clear from the ^{13}C spectrum, shown as the vertical projection in Fig. 9–22, that the single carbonyl in the precursor monomer FA-Ara **64** has generated a variety of carbonyl moieties in the polymer that are both saturated and unsaturated (groupings labeled **A-D**). The long-range correlations from the labeled carbonyl carbon to protons 2 or 3 bonds away are particularly diagnostic and provide evidence for *β*-*O*-4′, *β*-5′, 4-*O*-*β*′, and *β*-*β*′ products of the types shown in Fig. 9–23.

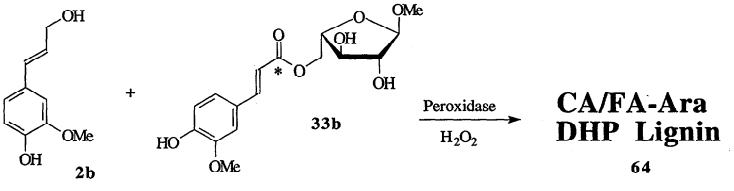


Fig. 9–21. Preparation of DHP **64**, a radical copolymer of coniferyl alcohol **2b** and γ-^{13}C-labeled FA-Ara **33b**.

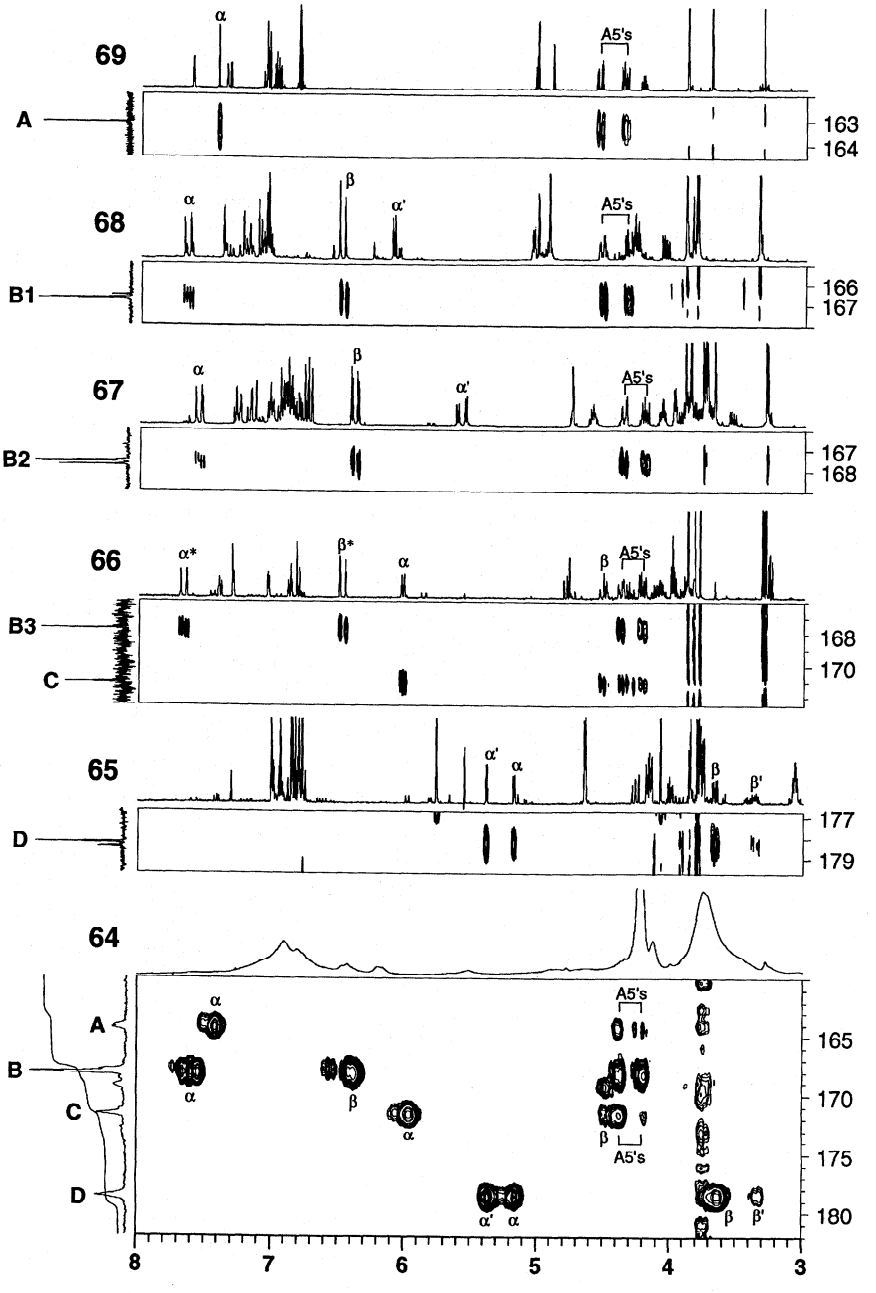


Fig. 9–22. Portion of inverse-detected long-range 2D C-H correlation NMR spectrum showing just the carbonyl carbon region. The 1D carbon and proton spectra along the axes are from quantitative 1D experiments. Peak groupings **A-D** are assigned to structures in Fig. 9–23. Authentication of assignments is shown via similar regions of the HMBC spectra of model compounds **65-69** (compounds as in Fig. 9–24).

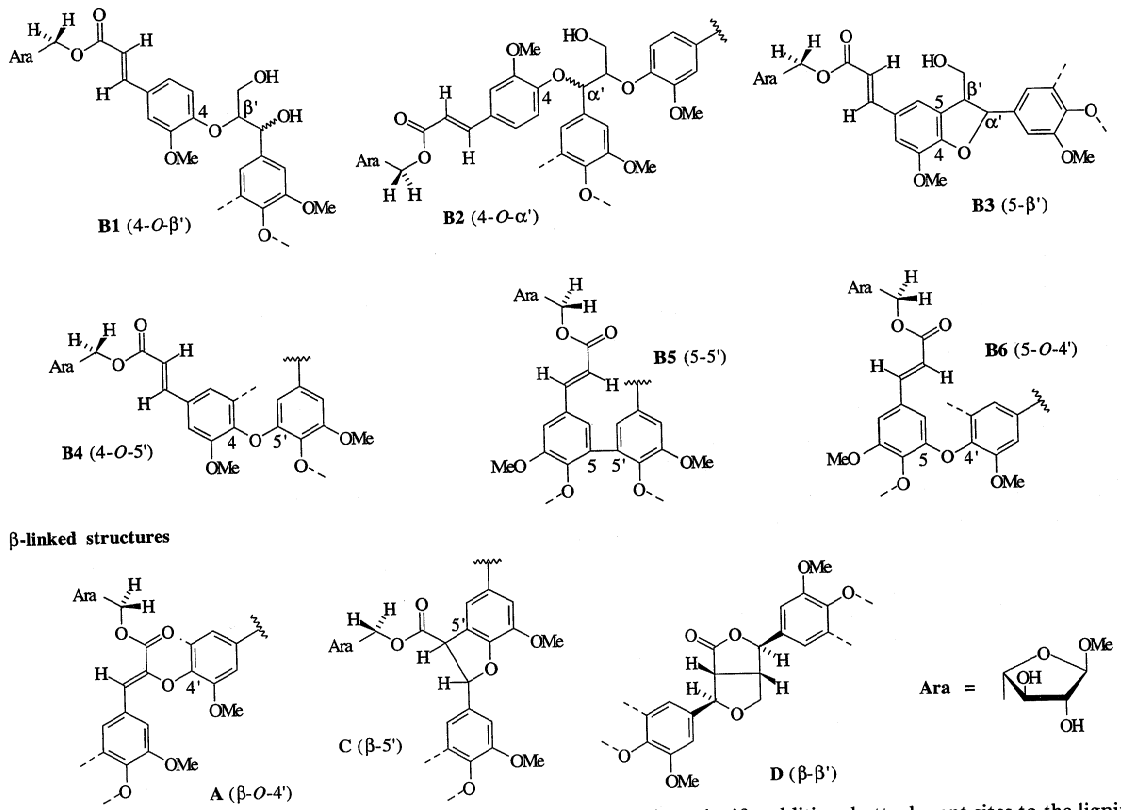


Fig. 9–23. Structures proposed for peaks **A, B, C**, and **D** of Fig. 9–22. The dotted lines signify additional attachment sites to the lignin polymer. Note that 'Ara' in these figures represents the arabinosyl moiety less the C-5, so that the C-5 protons can be shown explicitly, depicting their three-bond relationship to the Cγ carbonyl, and hence resulting in the long-range correlations shown in Fig. 9–22. Naming conventions are as for Fig. 9–19.

The major peak cluster, B (Fig. 9–22), shows that the double bond is intact (α and β protons at normal chemical shifts) and the correlation with the five-protons of the arabinosyl moiety indicates that the ferulic acid-arabinosyl ester linkage has remained intact. The multiplicity of peaks in this region is certainly due to the variety of substitutions possible on the aromatic ring, through the phenol or the 5-position as shown in structures **B1-B6** (Fig. 9–23), and to remote stereochemistry (e.g., the *threo* or *erythro* β-ether subunit attached at C4 in **B1**, or 4-*O*-α' **[B2]** vs. 4-*O*-β' **[B1]** substitution). The spectra (Fig. 9–22) from the α-ether models **67** and β-ether models **68** (Fig. 9–24) coincide with the major peaks in group **B**, suggesting that the two largest peaks in the DHP carbon spectrum are probably due to phenolic-etherified structures. It is important to recognize that structures **B1** and **B2** result from fundamentally different reaction pathways (Fig. 9–25): whereas 4-*O*-β' structures **B1** are formed as a direct result of radical coupling mechanisms and implicate FA-Ara directly in the enzymatically initiated dehydrogenation, 4-*O*-α' structures **B2** result from attack of the intact FA-Ara on quinone methide intermediates. Determination of the partitioning between these two pathways, which is not clear from this data, will be the subject of a future study using 4-labeled FA-Ara.

Peak cluster **A** also depicts structures with the arabinosyl moiety attached but retains only one (shifted) vinylic proton. It is quickly concluded that this represents the β-*O*-4′ structures shown for **A** in Fig. 9–23, as formed via the mechanism illustrated in Fig. 9–25. The observation of this β-linked product demonstrates that feruloyl moieties will, like coniferyl alcohol radi-

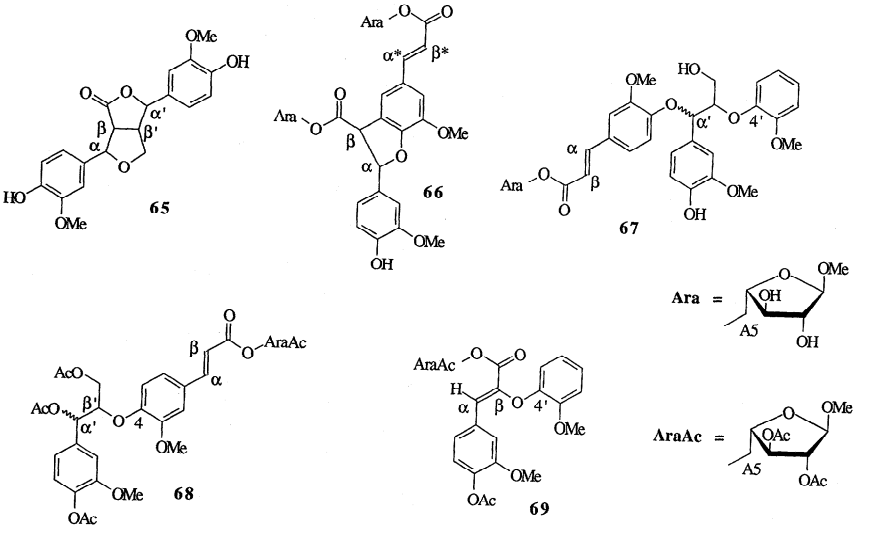


Fig. 9–24. Synthesized compounds **65-69** for authentication of the assignments made in Fig. 9–22. Naming conventions are as for Fig. 9–19. Note that compound **66** arises from coupling of two FA-Ara monomers and is not expected to be a prevalent structure in the DHP since cross-coupling of FA-Ara and coniferyl alcohol or the growing DHP is more likely. However, **66** models both **B3** and **C** structures reasonably well (see Fig. 9–22).

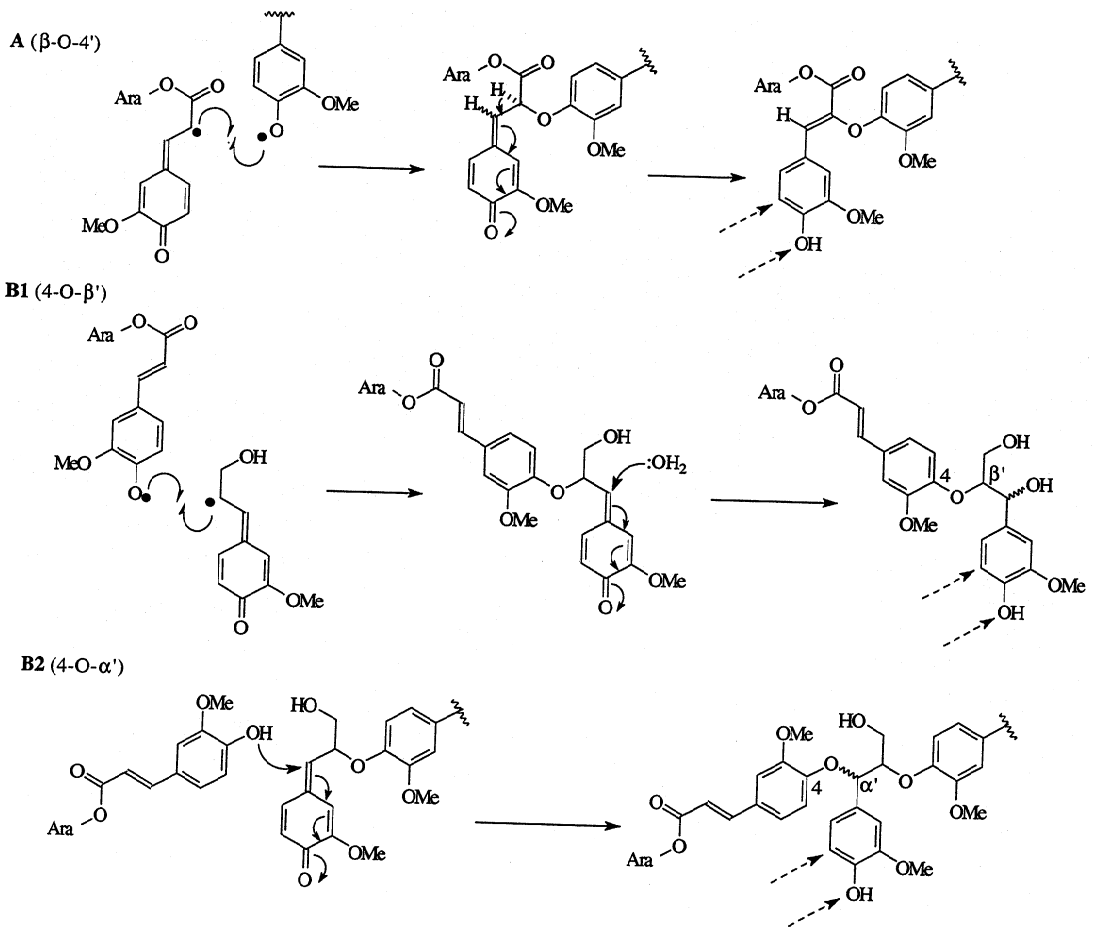
cals, couple efficiently at the β-position, on the sidechain. The difference in this instance is that the resulting quinone methide simply eliminates the acidic β-proton to form the conjugated structure, rather than trapping water or any other nucleophile to form an α-oxy product. This elimination mechanism was previously noted for coniferaldehyde (Connors et al., 1970). Interestingly, it is only the Z-isomer that is detected here (Fig. 9–22). This is in complete accord with observations made from the synthesis of compound **69**, a model for peaks **A**; only the Z-isomer resulted from an analogous quinone methide reaction, whereas both geometrical isomers resulted from a more reactive carbonium ion (Ralph et al., 1992b).

Peak cluster **C** again retains the arabinosyl moiety, but no longer the conjugated double bond. The correlation with protons at 5.95 and 4.48 ppm indicate a phenylcoumaran (β-5′) structure (Fig. 9–23), resulting from the mechanism in Fig. 9–25. Model **66** shows almost identical correlations (Fig. 9–22), confirming the assignment.

With peak cluster **D** it is clear from Fig. 9–22 that the arabinosyl moiety is no longer attached, and the correlations point to the half-lactone/half cyclic ether pinoresinolide product **D** represented in Fig. 9–23, derived from β-β coupling as shown mechanistically in Fig. 9–25. This further β-coupling product is excellent proof that coupling is taking place between the feruloyl moiety and the more predominant coniferyl alcohol radicals. It is also the only mechanism at this point that involves cleavage of the ester to eliminate the carbohydrate moiety.

As noted in more general terms above, of the products depicted in Fig. 9–23 only the 4-*O*-β′ product **B1** (corresponding to the **a/z** coupling product in Fig. 9–19), the 4-*O*-α′ product **B2**, and possibly the β-*O*-4′ product **A** (corresponding to the **c/x** product in Fig. 9–19) could regenerate ferulic acid (or logical derivatives) upon acidolysis, thioacidolysis, or high temperature alkaline solvolysis. The carbon plot shown on Fig. 9–22 was from a high resolution spectrum run under quantitative conditions. Examination of the integrals shows that cluster **A** represents 9% (and may give ferulic acid products) and cluster **B** 37%. While the major peaks of cluster **B** probably arise from phenolic α- or β-ethers that are not further condensed, there is a variety of other structures in this region that are condensed (structures **B3-B6** in Fig. 9–23, or the condensed structures indicated by the dashed bonds in **B1** and **B2**). We estimated (Ralph et al., 1992) from these spectra that $<40\%$ of the products from this reaction would be extractable by high-temperature base. Using high temperature base hydrolysis, it was experimentally determined that only 10% of the ferulic acid can be recovered from DHP **64**, increasing speculation that, if copolymerization is involved in the plant, the quantity of hydroxycinnamic acids measured by current procedures may severely underestimate their incorporation into the lignin/hydroxycinnamic acid polymer complex.

The other class of DHPs that can become valuable are those involving pre-formed bonds of the types expected (or to be tested) in the lignin/hydroxycinnamic acid complex. An example of this is lignin-*p*-coumaric acid γ-ester. As noted above, this ester cannot arise during the lignification and


A (β-O-4')
Ara
OMe
MeO
OH
B1 (4-O-β')
:OH2
4
β'
B2 (4-O-α')
HO
α'

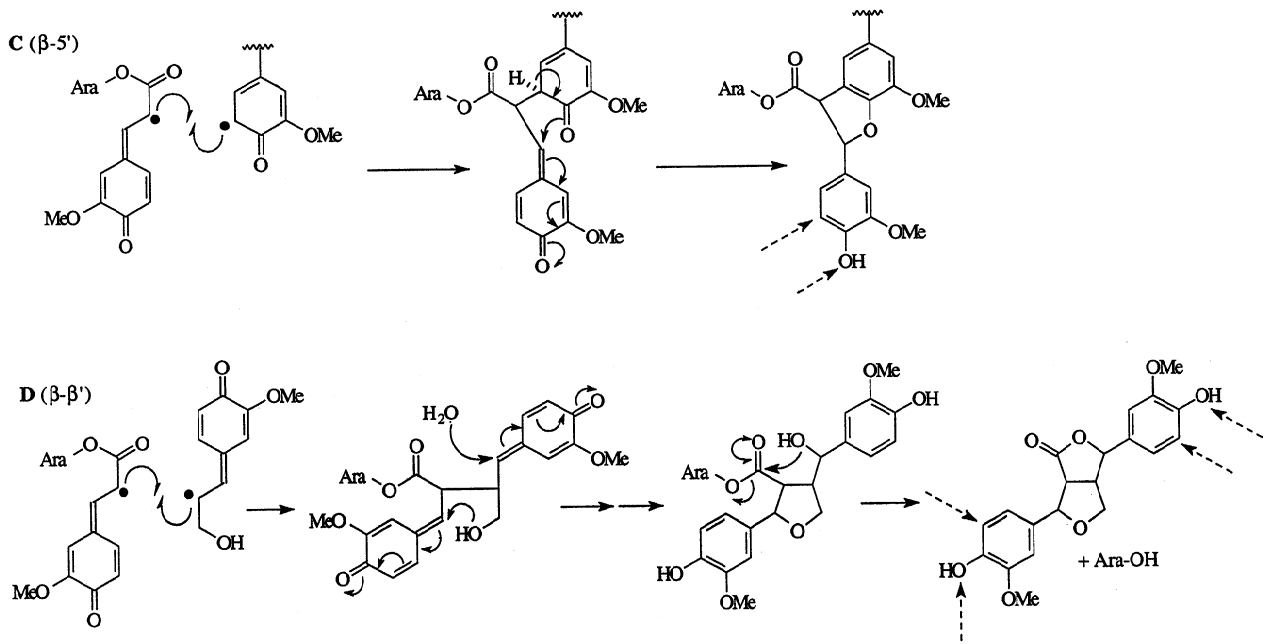


Fig. 9-25. Mechanisms for the formation of structures of types **A-D** (Fig. 9–23).

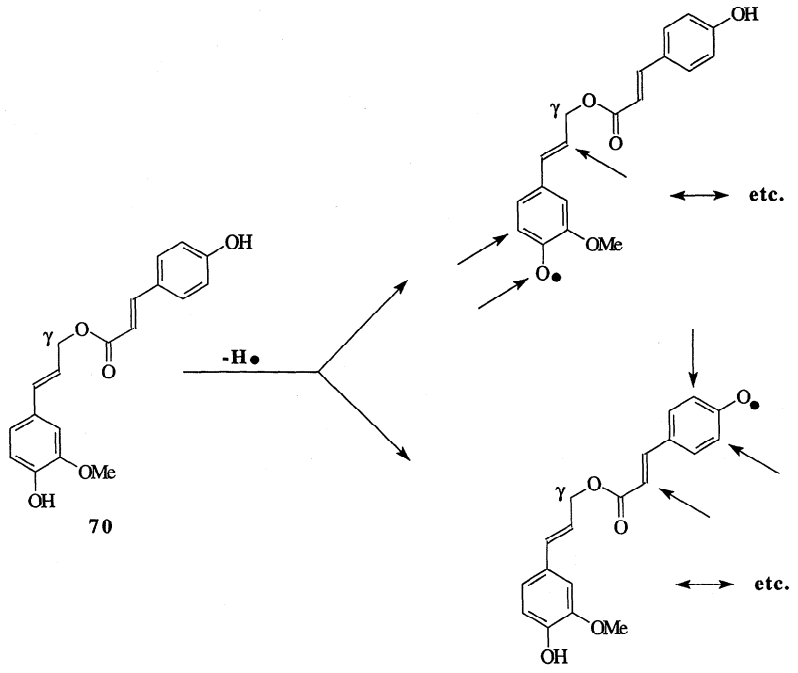


Fig. 9–26. Coniferyl *p*-coumarate, and its two derived 1-electron oxidation radicals. The arrows indicate the potential sites for radical coupling.

is unlikely to be formed by enzymatic reactions on the intact polymer. Consequently, the esterification of hydroxycinnamyl monomers to give esters such as **70** (Fig. 9–26), and their incorporation into the polymerization seems logical. As coniferyl *p*-coumarate **70** is a di-phenol, however, there is a whole range of possible products to be expected from in vitro 1-electron oxidation. Figure 9–26 illustrates the radical coupling available from H-abstraction of each phenolic OH. Synthesis of a DHP incorporating coniferyl *p*-coumarate should reveal which of these pathways are experimentally possible. If a plant really wants *p*-coumarate esters on lignin, (as opposed to this plethora of cross-linked structures), the phenol on the *p*-coumarate moiety may need to be protected, perhaps as a phenolic glycoside. The preparation and characterization of the DHP is in progress.

III. CONCLUSION

Characterization of plant tissues and cell wall isolates has involved NMR, FTIR, pyrolysis-MS, or pyrolysis-GC-MS (Reviewed in: Ralph & Hatfield, 1991), and chemical methods such as thioacidolysis, acidolysis, base hydrolysis, and elegant schemes such that of Lam et al. (1992). All of these methods have added substantially to the understanding of the importance of hydroxy-

cinnamic acids in plant cell walls. The methods of Lam et al. in particular have conclusively demonstrated that these structures are involved in cross-linking cell wall polymers. Little has been done in the context of this chapter, that is on the regiochemical characterization of these structures onto the lignin polymer. The concerted application of detailed model studies, using both low and high molecular weight models incorporating strategically placed ^{13}C labels to provide authenticated NMR data and insight into reaction possibilities, and the isolation and detailed NMR characterization of lignin/hydroxycinnamic acid/polysaccharide complexes should, in time, unambiguously answer many of these questions. At the same time there is scope for innovative design of degradative schemes that can answer specific regiochemical questions. The works of Lapierre et al. on thioacidolysis (which yields quantitative data on specific structural types), Lam et al. on proving the involvement of hydroxycinnamic acids in cross-linking, and Watanabe et al. on the DDQ method for determining the regiochemistry of ether attachment to lignins and carbohydrates, provide striking examples of the kinds of data that become available via well-planned schemes. Much of this work requires the use of model compounds to optimize reaction conditions and determine products. We foresee an increasing demand for model compounds that warrants the effort required to develop efficient syntheses and provide unambiguously assigned data.

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REFERENCES

Adler, E. 1977. Lignin chemistry—Past, present and future. Wood Sci. Technol. 11(3):169-218.

Atalla, R.H., and U.P. Agarwal. 1985. Raman microprobe evidence for lignin orientation in the cell walls of native woody tissue. Science 227(4687):636-638.

Bacic, A., P.J. Harris, and B.A. Stone. 1988. Structure and function of plant cell walls. p. 297–371. *In* The biochemistry of plants. Vol. 14. Academic Press, New York.

Bax, A., and G. Drobny. 1985. Optimization of two-dimensional homonuclear relayed coherence transfer NMR spectroscopy. J. Magn. Reson. 61(2):306–320.

Bax, A., and S. Subramanian. 1986. Sensitivity-enhanced two-dimensional heteronuclear shift correlation NMR spectroscopy. J. Magn. Reson. 67(3):565–569.

Braunschweiler, L., and R.R. Ernst. 1983. Coherence transfer by isotropic mixing: Application to proton correlation spectroscopy. J. Magn. Reson. 53(3):521–528.

Brunow, G., and K. Lundquist. 1991. On the acid-catalysed alkylation of lignins. Holzforschung 45(1):37–40.

Brunow, G., H. Peräkylä, and A. Tervilä-Wilo. 1989. The synthesis of hexameric β-aryl ether lignin model compounds. p. 249–252. *In* Proc. Int. Symp. of Wood and Paper Chemistry, 5th, North Carolina State Univ., Raleigh. 22–25 May 1989. TAPPI Press, Atlanta.

Brunow, G., J. Sipila, K. Lundquist, and S. Von Unge. 1988. The preparation of *threo* forms of β-*O*-4 type lignin model compounds. Cell. Chem. Technol. 22(2):191–199.

Brunow, G., and H. Wallin. 1981. Studies concerning the preparation of synthetic lignin. p. 125–127. *In* Proc. Ekman-Days/Int. Symp. Wood Pulping Chem., Stockholm, Sweden. 9–12 June 1981. SPCI, Stockholm, Sweden.

Buxton, D.R., and J.B. Russell. 1988. Lignin constituents and cell-wall digestibility of grass and legume stems. Crop Sci. 28(3):553–558.

Connors, W.J., C. Chen, and J.C. Pew. 1970. Enzymic dehydrogenation of the lignin model coniferaldehyde. J. Org. Chem. 35(6):1920–1924.

Davis, D.G., and A. Bax. 1985. Assignment of complex proton NMR spectra via two-dimensional homonuclear Hartmann-Hahn spectroscopy. J. Am. Chem. Soc. 107(9):2820–2821.

Delmer, D.P., and D.A. Stone. 1988. Biosynthesis of plant cell walls. p. 373–420. *In* The biochemistry of plants. Academic Press, New York.

Erickson, M., G.E. Miksche, and I. Somfai. 1973. Characterization of angiosperm lignins by degradation. II. Monocotyledons. Holzforschung 27(5):147–150.

Faix, O. 1986. Investigation of lignin polymer models (DHP's) by FTIR spectroscopy. Holzforschung 40(5):273–280.

Faix, O., and O. Beinhoff. 1988. FTIR spectra of milled wood lignins and lignin polymer models (DHP's) with enhanced resolution obtained by deconvolution. J. Wood Chem. Technol. 8(4):505–522.

Fengel, D., and G. Wegener. 1989. Wood. Chemistry, ultrastructure, reactions. Walter de Gruyter, New York.

Fletcher, H.G. 1963. The anomeric tri-*O*-benzoyl-D-arabinofuranosyl bromides. Methods in Carbohydr. Chem. 2:228–236.

Ford, C.W., and R.D. Hartley. 1989. GC/MS characterization of cyclodimers from *p*-coumaric and ferulic acids by photodimerization—a possible factor influencing cell wall biodegradability. J. Sci. Food Agric. 46(3):301–310.

Ford, C.W., and R.D. Hartley. 1990. Cyclodimers of *p*-coumaric and ferulic acids in the cell walls of tropical grasses. J. Sci. Food Agric. 50(1):29–43.

Fry, S.C. 1982. Phenolic components of the primary cell wall. Biochem. J. 203:493–504.

Fry, S.C. 1984. Incorporation of ^{14}C cinnamate into hydrolase-resistant components of the primary cell wall: feruloylated disaccharides of D-galactose and L-arabinose from spinach polysaccharide. Phytochemistry 23:59–64.

Fry, S.C. 1985. Primary cell wall metabolism. Oxford Surv. of Plant Molec. Cell Biol. 2:1–42.

Fry, S.C., and J.C. Miller. 1989. Toward a working model of the growing plant cell wall. Phenolic cross-linking reactions in the primary cell walls of dicotyledons. p. 33–46. *In* N.G. Lewis and M.G. Paice (ed.) Plant cell wall polymers, biogenesis and biodegradation. ACS Symp. Ser. 399. Am. Chem. Soc., Washington, DC.

Gagnaire, D., and D. Robert. 1977. A polymer model of lignin (D.H.P.) carbon-13 selectively labeled at the benzylic positions: synthesis and NMR study. Makromol. Chem. 178(5): 1477–1495.

Glasser, W.G., C.A. Barnett, and Y. Sano. 1983. Classification of lignins with different genetic and industrial origins. J. Appl. Polym. Sci.: Appl. Polym. Symp., 37(Proc. Cellul. Conf., 9th, 1982, Part 1):441–460.

Gordon, A.J. 1975. Comparison of chemical and physical properties of alkali lignins from grass and lucerne hays before and after digestion by sheep. J. Sci. Food Agric. 26(10):1551–1559.

Goring, D.A.I. 1989. The lignin paradigm. p. 2–10. *In* W.G. Glasser and S. Sarkanen (ed.) Lignin: Properties and materials. Am. Chem. Soc., Washington, DC.

Harkin, J.M. 1967. Lignin—A natural polymeric product of phenol oxidation. p. 243–321. *In* W.I. Taylor and A.R. Battersby (ed.) Oxidative coupling of phenols. Marcel Dekker, New York.

Harkin, J.M. 1973. Lignin. p. 323–373. In G.W. Butler (ed.) Chemistry and biochemistry of herbage. Vol. 1. Academic Press, London.

Hartley, R.D., and C.W. Ford. 1989. Phenolic constituents in plant cell walls and wall biodegradability. p. 137–139. *In* N.G. Lewis and M.G. Paice (ed.) Plant cell wall polymers, biogenesis and biodegradation. ACS Symp. Ser. 399. Am. Chem. Soc., Washington, DC.

Hartley, R.D., and E.C. Jones. 1978. Phenolic components and degradability of the cell walls of the brown midrib mutant, bm3, of *Zea mays*. J. Sci. Food Agric. 29(9):777–782.

Hartley, R.D., W.H. Morrison, F. Balza, and G.H.N. Towers. 1990a. Substituted truxillic and truxinic acids in cell walls of *Cynodon dactylon*. Phytochemistry 29(12):3699–3703.

Hartley, R.D., W.H. Morrison, D.S. Himmelsbach, and W.S. Borneman. 1990b. Cross-linking of cell wall phenolic arabinoxylans in graminaceous plants. Phytochemistry 29(12):3705–3709.

Hartley, R.D., F.R. Whatley, and P.J. Harris. 1988. 4,4′-Dihydroxytruxillic acid as a component of cell walls of *Lolium multiflorum*. Phytochemistry 27(2):349–351.

Hatfield, R.D., R.F. Helm, and J. Ralph. 1991. Synthesis of methyl-5-*O*-*trans*-feruloyl-α-L-arabinofuranoside and its use as a substrate to assess feruloyl esterase activity. Anal. Biochem. 194(1):25–33.

Hatfield, R.D., B.A. Uicker, R.F. Helm, and J. Ralph. 1993. Isolation and characterization of hydroxycinnamoyl esterases from *Humicola insolens*. Plant Cell Physiol. (In press.)

He, L., and N. Terashima. 1989. Formation and structure of lignin in monocotyledons. II. Deposition and distribution of phenolic acids and their association with cell wall polymers in rice plants (*Oryza sativa*). Mokuzai Gakkaishi 35(2):123–129.

He, L., and N. Terashima. 1990. Formation and structure of lignin in monocotyledons. III. Heterogeneity of sugarcane (*Saccharum officinarum* L.) lignin with respect to the composition of structural units in different morphological regions. J. Wood Chem. Technol. 10(4):435–459.

Helm, R.F., and J. Ralph. 1992a. Lignin—hydroxycinnamyl model compounds related to forage cell wall structure. Part 1. Ether-linked structures. J. Agric. Food Chem. 40(11):2167–2175.

Helm, R.F., and J. Ralph. 1992b. Lignin—hydroxycinnamyl model compounds related to forage cell wall structure. Part 2. Ester-linked structures. J. Agric. Food Chem. (In press.)

Helm, R.F., J. Ralph, and L. Andersen. 1991. Regioselective protection strategies for xylopyranosides. J. Org. Chem. 56(25):7015–7021.

Helm, R.F., J. Ralph, and R.D. Hatfield. 1992. Synthesis of feruloylated and *p*-coumaroylated methyl glycosides. Carbohydr. Res. 229:183–194.

Higuchi, T., Y. Ito, M. Shimada, and I. Kawamura. 1967. Chemical properties of milled wood lignin of grasses. Phytochemistry 6(11):1551–1556.

Himmelsbach, D.S., and F.E. Barton. 1980. Carbon-13 nuclear magnetic resonance of grass lignins. J. Agric. Food Chem. 28(6):1203–1208.

Hosoya, S., K. Kanazawa, H. Kaneko, H. Nakano, and J. Nakano. 1980. Synthesis of guaiacylglycerol-β-guaiacyl ether. Mokuzai Gakkaishi 26(2):118–121.

Hyatt, J.A. 1987. Synthesis of some tetrameric lignin model compounds containing β-O-4 and 5,5′-interunit linkages. Holzforschung 41(6):363–370.

Iiyama, K., T.B.T. Lam, and B.A. Stone. 1990. Phenolic acid bridges between polysaccharides and lignin in wheat internodes. Phytochemistry 29(3):733–737.

Ishii, T. 1991. Acetylation at *O*-2 of arabinofuranose residues in feruloylated arabinoxylan from bamboo shoot cell walls. Phytochemistry 30(7):2317–2320.

Ishii, T., and T. Hiroi. 1990a. Linkage of phenolic acids to cell-wall polysaccharides of bamboo shoot. Carbohydr. Res. 206(2):297–310.

Ishii, T., and T. Hiroi. 1990b. Isolation and characterization of feruloylated arabinoxylan oligosaccharides from bamboo shoot cell walls. Carbohydr. Res. 196:175–183.

Ishii, T., T. Hiroi, and J.R. Thomas. 1990. Feruloylated xyloglucan and *p*-coumaroyl arabinoxylan oligosaccharides from bamboo shoot cell walls. Phytochemistry 29(6):1999–2003.

Jeffries, T.W. 1990. Biodegradation of lignin-carbohydrate complexes. Biodegradation 2:163–176.

Jung, H.G., and G.C. Fahey. 1983. Nutritional implications of phenolic monomers and lignin: A review. J. Anim. Sci. 57(1):206–219.

Jung, H.G., and J. Ralph. 1990. Phenolic-carbohydrate complex in plant cell walls and their effect on lignocellulose utilization. p. 173–182. *In* D.E. Akin et al. (ed.) Microbial and plant opportunities to improve lignocellulose utilization by ruminants. Elsevier Sci. Publ. Co., New York.

Kamaya, Y., F. Nakatsubo, and T. Higuchi. 1980. Synthesis of a trimeric lignin model compound composed of β-*O*-4 and syringaresinol substructures. Mokuzai Gakkaishi 26(7):471–475.

Kamisaka, S., S. Takeda, K. Takahashi, and K. Shibata. 1990. Diferulic and ferulic acid in the cell wall of *Avena* coleoptiles—their relationships to mechanical properties of the cell wall. Physiol. Plant. 78(1):1–7.

Katayama, T., S. Kawai, M. Sogo, and T. Higuchi. 1987. Degradation of guaiacylglycerol-α-vanillyl alcohol-β-vanillin diether, a lignin substructure model containing a non-cyclic benzyl aryl ether, by *Fusarium solani* M-13-1. Mokuzai Gakkaishi 33(6):503–510.

Kato, A., J. Azuma, and T. Koshijima. 1983. A new feruloylated trisaccharide from bagasse. Chem. Lett. 137–140.

Kato, A., J. Azuma, and T. Koshijima. 1987. Isolation and identification of a new feruloylated tetrasaccharide from bagasse lignin-carbohydrate complex containing phenolic acid. Agric. Biol. Chem. 51(6):1691–1693.

Kato, Y., and D.J. Nevins. 1985. Isolation and identification of *O*-(5-*O*-feruloyl-α-L-arabinofuranosyl)-(1→3)-*O*-β-D-xylopyranosyl-(1→4)-D-xylose as a component of *Zea* shoot cell-walls. Carbohydr. Res. 137:139–150.

Kirk, T.K., and G. Brunow. 1988. Synthetic carbon-14-labeled lignins. Methods Enzymol. 161 (Biomass, Pt.B):65–73.

Lam, T.B.T., K. Iiyama, and B. Stone. 1990. Distribution of free and combined phenolic acids in wheat internodes. Phytochemistry 29(2):429–433.

Lam, T.B.T., K. Iiyama, and B. Stone. 1991. Quantitative determination of lignin-polysaccharide associations in wheat internode cell walls: Ferulic acid bridges. p. 29–33. *In* Proc. Int. Symp. of Wood and Paper Chemistry, 6th, Melbourne, Australia. 29 Apr.–3 May 1991. Australian Pulp and Paper Ind. Tech. Assoc. (APPITA), Parkville, Victoria.

Lam, T.B.T., K. Iiyama, and B. Stone. 1992. Cinnamic acid bridges between cell wall polymers in wheat and phalaris internodes. Phytochemistry 31:1179–1183.

Landucci, L.L., S.A. Geddes, and T.K. Kirk. 1981. Synthesis of carbon-14-labeled 3-methoxy-4-hydroxy-α-(2-methoxyphenoxy)-β-hydroxypropiophenone, a lignin model compound. Holzforschung 35(2):67–70.

Lewis, N.G., J. Newman, G. Just, and J. Ripmeister. 1987. Determination of bonding patterns of carbon-13 specifically enriched dehydrogenatively polymerized lignin in solution and solid state. Macromolecules 20(8):1752–1756.

Lyr, H. 1957. Über die an der Ligninbildung beteilgten Fermentsysteme. Naturwissenschaften 44(7):235.

Monties, B. 1991. Recent advances in structural and biosynthetic variability of lignins. p. 113–123. *In* Proc. Int. Symp. of Wood and Pulping Chemistry, 6th, Melbourne, Australia. 29 Apr.–3 May 1991. Australian Pulp and Paper Ind. Tech. Assoc. (APPITA), Parkville, Victoria.

Mueller-Harvey, I., R. Hartley, P.J. Harris, and E.H. Curzon. 1986. Linkage of *p*-coumaryl and feruloyl groups to cell wall polysaccharides of barley straw. Carbohydr. Res. 148:71–85.

Nakamura, Y., and T. Higuchi. 1976. Ester linkage of *p*-coumaric acid in bamboo lignin. Holzforschung 30(6):187–191.

Nakamura, Y., and T. Higuchi. 1978a. Ester linkage of *p*-coumaric acid in bamboo lignin. III. Dehydrogenative polymerization of coniferyl *p*-hydroxybenzoate and coniferyl *p*-coumarate. Cell. Chem. Technol. 12(2):209–221.

Nakamura, Y., and T. Higuchi. 1978b. Ester linkage of *p*-coumaric acid in bamboo lignin. II. Syntheses of coniferyl *p*-hydroxybenzoate and coniferyl *p*-coumarate as possible precursors of aromatic acid esters in lignin. Cell. Chem. Technol. 12(2):199–208.

Nakatsubo, F. 1981. Enzymic dehydrogenation of *p*-coumaryl alcohol and syntheses of oligolignols. Wood Res. 67:59–118.

Nakatsubo, F., and T. Higuchi. 1980a. Synthesis of trimeric lignin model compound composed of phenylcoumaran and β-1 structures. Mokuzai Gakkaishi 26(2):107–111.

Nakatsubo, F., and T. Higuchi. 1980b. Synthesis of trimeric lignin model compounds composed of phenylcoumaran and β-*O*-4 structures. Makuzai Gakkaishi 26(1):31–36.

Nakatsubo, F., K. Sato, and T. Higuchi. 1975. Synthesis of guaiacylglycerol-β-guaiacyl ether. Holzforschung 29(5):165–168.

Namba, H., F. Nakatsubo, and T. Higuchi. 1980. Synthesis of trimeric lignin model compound composed of β-*O*-4 and β-1 structures. Mokuzai Gakkaishi 26(6):426–431.

Nimz, H., I. Mogharab, and H.D. Luedemann. 1974. Carbon-13-NMR spectra of lignins. 3. Comparison of spruce lignin with synthetic lignin according to Freudenberg. Makromol. Chem. 175(9):2563–2575.

Nimz, H.H., D. Robert, O. Faix, and M. Nemr. 1981. Carbon-13 NMR spectra of lignins. 8. Structural differences between lignins of hardwoods, softwoods, grasses and compression wood. Holzforschung 35(1):16–26.

Ragauskas, A.J. 1991a. Synthetic efforts directed at lignin model oligomers. Paper CELL-26. *In* Proc. Am. Chem. Soc. Natl. Meet., 201st, Atlanta, GA. 14–19 Apr. 1991. Am. Chem. Soc., Washington, DC.

Ragauskas, A.J. 1991b. Fundamental investigations of brightness reversion in mechanical pulp. Paper OR-HZ. *In* Proc. Canadian Chem. Conf., 74th, Hamilton, ON. 2–6 June 1991. Chem. Inst. of Canada (CIC), Ottawa, ON.

Ralph, J. 1982a. Reactions of lignin model quinonemethides and NMR studies of lignins. Ph.D. diss. Univ. of Wisconsin, Madison (Diss. Abstr. 82-26987).

Ralph, J. 1982b. Stereochemical aspects of lignin and lignin model reactions. p. 153–155. *In* Proc. Canadian Wood Chemistry Symp., Niagara Falls, ON, Canada. 13–15 Sept. 1982. Chem. Inst. of Canada (CIC) and Canadian Pulp and Paper Assoc. (CPPA), Ottawa, ON.

Ralph, J. 1988. NMR of lignin model compounds: Application of long-range C-H correlations through oxygen. Holzforschung 42(4):273–275.

Ralph, J., R.M. Ede, and A.L. Wilkins. 1986. Synthesis of trimeric lignin model compounds composed of β-aryl ether and phenylcoumaran structures. Holzforschung 40(1):23–30.

Ralph, J., and R.D. Hatfield. 1991. Pyrolysis-GC-MS characterization of forage materials. J. Agric. Food Chem. 39(8):1426–1437.

Ralph, J., and R.F. Helm. 1991. Rapid proton NMR method for determination of *threo:erythro* ratios in lignin model compounds and examination of reduction stereochemistry. J. Agric. Food Chem. 39(4):705–709.

Ralph, J., R.F. Helm, S. Quideau, and R.D. Hatfield. 1992a. Lignin-feruloyl ester cross-links in grasses. Part 1. Incorporation of feruloyl esters into coniferyl alcohol dehydrogenation polymers. J. Chem. Soc., Perkin Trans. I. 2961–2969.

Ralph, J., R.F. Helm, and S. Quideau. 1992b. Lignin-feruloyl ester cross-links in grasses. Part 2. Model compound syntheses. J. Chem. Soc., Perkin Trans. I. 2971–2980.

Ralph, J., and C. Rodger. 1991. NMR of lignin model trimers, or why you will never find crystalline regions in lignin. p. 59–64. *In* Proc. Int. Symp. of Wood and Paper Chemistry, 6th, Melbourne, Australia. 29 Apr.–3 May 1991. Australian Pulp and Paper Ind. Tech. Assoc. (APPITA), Parkville, Victoria.

Ralph, J., and A.L. Wilkins. 1985. Rapid NMR method for determination of *threo:erythro* ratios in lignin model compounds. Holzforschung 39(6):341–344.

Ralph, J., and R.A. Young. 1981. Synthesis of the lignin model compounds *threo*-guaiacylglycerol-β-guaiacyl ether and *threo*-veratrylglycerol-β-guaiacyl ether. Holzforschung 35(1):39–41.

Ralph, J., and R.A. Young. 1983. Stereochemical aspects of addition reactions involving lignin model quinone methides. J. Wood Chem. Technol. 3(2):161–181.

Robert, D.R., and G. Brunow. 1984. Quantitative estimation of hydroxyl groups in milled wood lignin from spruce and in a dehydrogenation polymer from coniferyl alcohol using carbon-13 NMR spectroscopy. Holzforschung 38(2):85–90.

Sarkanen, K.V., and C.H. Ludwig. 1971. Lignins, occurrence, formation, structure, and reactions. Wiley Interscience, New York.

Scalbert, A., B. Monties, J.Y. Lallemand, E. Guittet, and C. Rolando. 1985. Ether linkage between phenolic acids and lignin fractions from wheat straw. Phytochemistry 24(6):1359–1362.

Scalbert, A., B. Monties, C. Rolando, and A. Sierra-Escudero. 1986. Formation of ether linkage between phenolic acids and Gramineae lignin: A possible mechanism involving quinone methides. Holzforschung 40(3):191–195.

Sharma, A., J. Brillouet, A. Scalbert, and B. Monties. 1986. Studies on a brittle stem mutant of rice, *Oryza sativa* L.; characterization of lignin fractions, associated phenolic acids and polysaccharides from rice stem. Agronomie 6(3):265–271.

Shimada, M., T. Fukuzuka, and T. Higuchi. 1971. Ester linkages of *p*-coumaric acid in bamboo and grass lignins. Tappi 54(1):72–78.

Sipilä, J. 1990. On the reactions of quinone methide intermediates during lignin biosynthesis. A study with model compounds. Academic Diss., Univ. of Helsinki. Ann. Acad. Sci. Fennicae, Ser. A II. Chemica.

Sipilä, J., and G. Brunow. 1991. On the reactions of quinone methide intermediates during lignin biosynthesis. p. 297–304. *In* Proc. Int. Symp. of Wood and Pulping Chemistry, 6th, Melbourne, Australia. 29 Apr.–3 May 1991. Australian Pulp and Paper Ind. Tech. Assoc. (APPITA), Parkville, Victoria.

Smith, D.C.C. 1955. Ester groups in lignin. Nature (London) 176:267–268.

Smith, M.M., and R.D. Hartley. 1983. Occurrence and nature of ferulic acid substitution of cell-wall polysaccharides in graminaceous plants. Carbohydr. Res. 118:65–80.

Taiz, L. 1984. Plant cell expansion: Regulation of cell wall mechanical properties. Ann. Rev. Plant Physiol. 35:585–667.

Tanahashi, M., T. Aoki, and T. Higuchi. 1982. Dehydrogenative polymerization of monolignols by peroxidase and hydrogen peroxide in a dialysis tube. II. Estimation of molecular weights by thermal softening method. Holzforschung 36(3):117–122.

Tanahashi, M., T. Aoki, and T. Higuchi. 1981. Dehydrogenative polymerization of monolignols by peroxidase and hydrogen peroxide in a dialysis tube. III. Formation of lignin-carbohydrate complexes (LCCs). Mokuzai Gakkaishi 27(2):116–124.

Tanahashi, M., and T. Higuchi. 1981. Dehydrogenative polymerization of monolignols by peroxidase and hydrogen peroxide in a dialysis tube. I. Preparation of highly polymerized DHPs. Wood Res. 67:29–42.

Tollier, M., C. Lapierre, B. Monties, C. Francesch, and C. Rolando. 1991. Structural variations in synthetic lignins (DHPs) according to the conditions of their preparation. Vol. 2. p. 35–40. *In* Proc. Int. Symp. of Wood and Paper Chemistry, 6th, Melbourne, Australia. 29 Apr.–3 May 1991. Australian Pulp and Paper Ind. Tech. Assoc. (APPITA), Parkville, Victoria.

Watanabe, T., T. Imamura, T. Kohshijima, and M. Karina. 1991. Structural analysis of lignin-carbohydrate complexes from Albizia Falcata Backer. Vol. 1. p. 275–279. *In* Proc. Int. Symp. of Wood and Paper Chemistry, 6th, Melbourne, Australia. 29 Apr.–3 May 1991. Australian Pulp and Paper Inc. Tech. Assoc. (APPITA), Parkville, Victoria.

Watanabe, T., J. Ohnishi, Y. Yamasaki, S. Kaizu, and T. Koshijima. 1989. Binding-site analysis of the ether linkages between lignin and hemicelluloses in lignin-carbohydrate complexes by DDQ oxidation. Agric. Biol. Chem. 53(8):2233–2252.

Yamamoto, E., G.H. Bokelman, and N.G. Lewis. 1989. Phenylpropanoid metabolism in cell walls, An overview. p. 33–46. *In* N.G. Lewis and M.G. Paice (ed.) Plant cell wall polymers, biogenesis and biodegradation. ACS Symp. Ser. 399. Am. Chem. Soc., Washington, DC.